[GANN, 50; 111~119; June, 1959]

ON A SUBSTANCE ISOLATED FROM CERTAIN HUMAN MALIGNANT TUMORS PROMOTING MITOSIS *IN VITRO**

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One of the most characteristic properties of malignant tumors is their uncontrolled accelerated growth. The present biochemical concept[11),16),18)] is inclined to ascribe the principal part of this phenomenon to an abnormally increased DNA synthesis. However it is self-evident that increased DNA synthesis alone cannot result in cell division.[12)] For the completion of cell division the participation of another mechanism —mitosis—is necessary. Indeed, the frequent appearance of mitotic figures is one of the microscopic peculiarities of malignant tissues. This fact suggests the existence

Table 1. Tumors utilized for preparations.

Case	Age	Sex	Clinical course	Source	Location	Histological diagnosis	Weight (g)
1	29	♂	Since ca. 6mo., l. testis gradually enlarged. No metastasis.	Operation	L. testis	Terato-carcinoma	250
2	28	♂	Since childhood, r. testicular tumor. Since a year enlarged. No metastasis.	Operation	R. testis	Terato-carcinoma	350
3	28	♂	Since a year. r. testicular tumor. advanced metastasis.	Operation	R. testis	Seminoma	90
4	17	♂	1 yr. ago, extirpated lower abd. tumor, recurred. Since 6 mo. marked liver metastasis.	Necropsy	Liver (metastasis)	Seminoma	200
5	1	♂	Since 6 mo., l. testicular tumor. No metastasis.	Operation	L. testis	Terato-carcinoma	40
6	25	♂	Cryptorchism (l.) operated under diagnosis of ileus. No metastasis.	Operation	L. testis in abdominal cavity	Seminoma	360
7	66	♀	Since ca. 30 yrs., l. lower abdominal tumor. Since 6 mo. rapidly enlarged.	Operation	Abdominal wall	Fibrosarcoma	338
8	34	♂	Since 8 mo. lower abdominal tumor, extirpated 5 mo. ago. Since 2 mo. large tumor recurred.	Operation	Retro-peritoneum	Leiomyo-sarcoma	780

*The expenses for this study were defrayed by a grant for the Scientific Researches from the Ministry of Education.

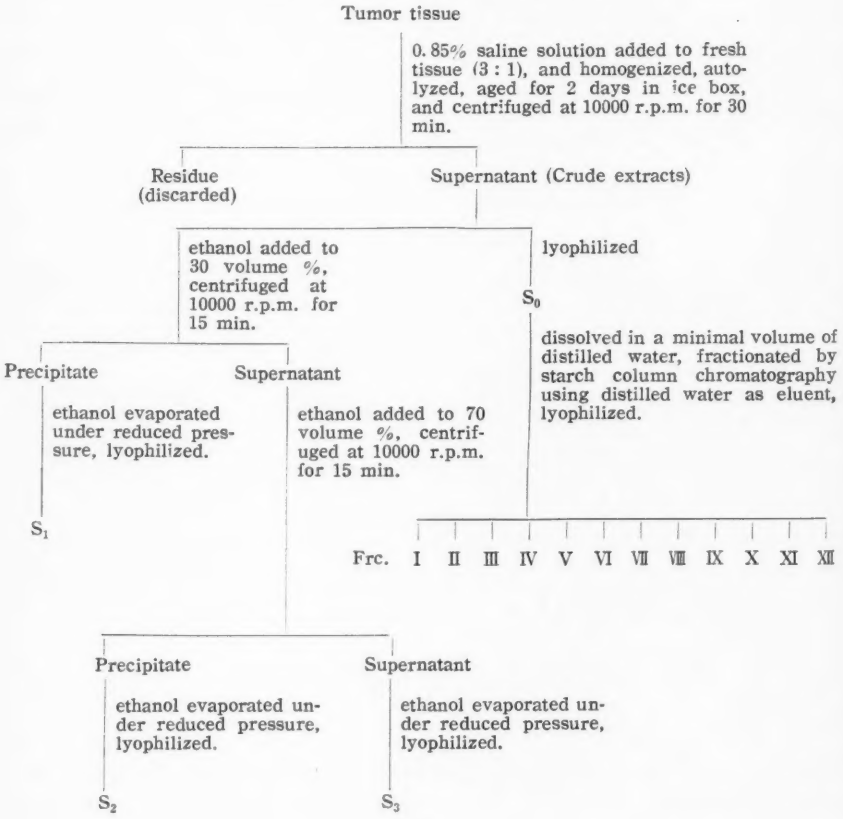
of some mitosis promoting substance (or substances) produced in malignant tissues themselves. The present study was undertaken to detect such a substance.

Material and Method

Fresh specimens of rapidly growing human malignant tumors were used, all but one being obtained by surgical operation. Data concerning the origin of the material used are briefly summarized in Table 1. Most of material was testicular tumor, in which, as is well known, especially rapid growth is noted. Extraction and fractionation was made by the methods indicated in Table 2.

For the evaluation of the mitosis promoting activity, Bullough's method[3),4)] was used. Adult male mice aged 3 to 6 months of ddO or C3H/HeN (courtesy of Prof. H. Sato) were used exclusively. The mice were killed by cervical dislocation

Table 2. Preparation of the mitosis promoting substances from rapidly growing tumors of man.



and immediately after death the thin peripheral region was detached from each ear and cut into 2-4 small pieces of approximately 2.5×5.0mm. Each piece was marked for identification of its origin.

For incubations Warburg flasks with 2 side-arms were used. The main chamber contained 4 ml of the culture solution, the constitution of which is shown in Table 3.

Table 3. Medium

Standard solution		
1) Saline solution :		9 Parts
0.90%	NaCl	100
1.15%	KCl	4
1.22%	$CaCl_2$	3
2.11%	KH_2PO_4	3
3.82%	$MgSO_47H_2O$	1
1.30%	$NaHCO_3$	3
2) 0.1M phosphate buffer (pH 7.4) :		1 Part
17.8gr	$NaHPO_42H_2O$	
20ml	N HCl	
diluted to a litre with aq. dest.		

To the above standard solution glucose was added at the final concentration 0.012M.

One side-arm contained the test material, 2mg of which had been dissolved in 0.2 ml distilled water. The other side-arm contained 0.016mg colchicine in 0.2 ml physiological saline. The one side-arm of the control flask contained 0.2 ml of distilled water instead of the test material. Two to four fragments, obtained from different animals, were put in the main chamber of the flasks. The flasks were then kept at 38°C and gently rocked, with air as the gas phase. If the ear fragments floated on the surface of the medium at the beginning of incubation, they were made to sink by shaking. After one hour, all the mitoses going on at the beginning of the experiments were presumed to have entered the anaphase. The colchicine and the test materials were then tipped into the main vessel. The incubation was continued for a further four hours, during which time the colchicine arrested all the newly developing mitoses at the metaphase. The ear fragments were taken out and fixed in Bouin's solution. Paraffin serial sections of 7μ thickness were made and stained with Mayer's haematoxylin.

The number of mitoses in the Malpighian layers, arrested at metaphase, were counted along a 10cm length of stained epidermis.

In a few experiments, the average number of mitoses found per unit length (1cm) of section, even in control experiments, showed considerable difference in relation to origin. The rejection limit ($p<0.05$) was, therefore, calculated. It was estimated to

be 24 (upper limit) and O (lower limit) by average values of mitoses found in a 1cm length of epidermis of the sections in 284 control experiments. Data of all the experiments, in which the average value of mitoses of controls exceeded the above rejection limit, were discarded.

Results

1) The results concerning the activity of each fraction obtained by starch column chromatography are summarized in Figs. 1-4. Fraction 6 in cases 1, 3 and 4 and fraction 5 in case 2, were found to promote the mitosis considerably, while the action of other fractions was rather inhibitory. Whether the effective substance in fraction 6 of cases 1, 3 and 4 and fraction 5 of case 2 is the same, could not be decided in our experiments, though it seems very likely.

2) Since the yields of the 6th (and 5th) fractions in fractionation by starch column chromatography are too scanty to allow further analysis, fractionation with ethanol was attempted. The results are reported below.

Fractions S_0, S_1, S_2 and S_3 from cases 5 to 8 and from normal bovine testis were

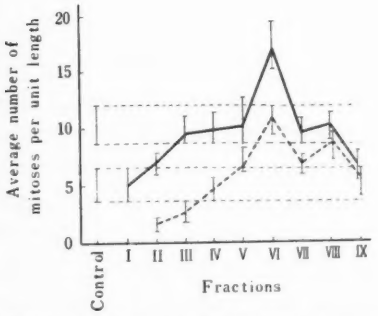


Fig. 1. Effect of individual fractions of the crude extracts from a teratocarcinoma of testis (case 1), fractionated by starch column chromatography.

The average number of mitoses arrested by colchicine in unit length (1cm) of sections 7μ thick of ear epidermis, incubated for 4 hrs. at 38°C in the culture medium, were plotted for each fraction. Standard deviation shown in the chart was calculated from 10 estimations. Solid line: mouse a, dotted line: mouse b.

The activity of the 10, 11 and 12th fractions was not examined, because the yield of these fractions was too scanty to alow examination.

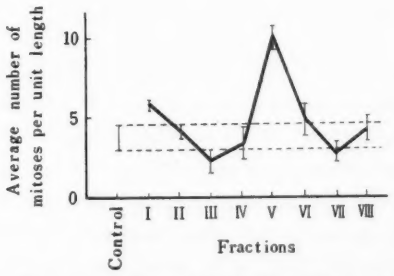


Fig. 2. Effect of individual fractions of the crude extracts from a teratocarcinoma of testis (case 2), fractionated by starch column chromatography.

The average number of mitoses arrested by colchicine in unit length (1cm) of sections of ear epidermis 7μ thick of a mouse, incubated for 4 hrs. at 38°C in the culture medium, were plotted for each fraction. The standard deviation shown in the chart was calculated from 10 estimations.

The activity of fractions 9, 10, 11 and 12 was not examined because the yield of these fractions was too scanty to allow examination.

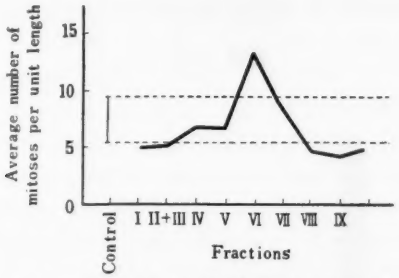


Fig. 3. Effect of individual fractions of crude extracts from a seminoma of testis (case 3), fractionated by starch column chromatography.

The average number of mitoses arrested by colchicine in unit length (1cm) of sections of ear epidermis 7μ thick of 5 mice, incubated for 4 hrs. at 38°C in the culture medium, were plotted for each fraction. The standard deviation of the control shown in the chart was calculated from 10 estimations.

The activity of fractions 10, 11 and 12 was not examined, because the yield of these fractions was too scanty to allow examination.

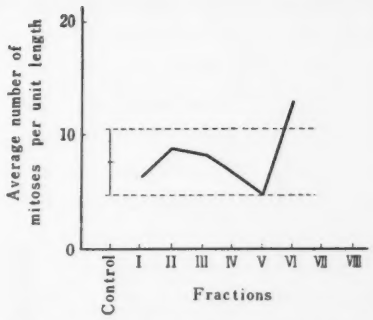


Fig. 4. Effect of individual fractions of the crude extracts from a seminoma of testis (case 4), fractionated by starch column chromatography.

The average number of mitoses arrested by colchicine in unit length (1cm) of sections of ear epidermis 7μ thick of 4 mice, incubated for 4 hrs. at 38°C in the culture medium, were plotted for each fraction. The standard deviation of the control shown in the chart was calculated from 10 estimations.

The activity of the 7–12th fractions was not examined because the yield of these fractions was too scanty to alow examination.

tested by Bullough's method (Figs. 5, 6, 7). The mitotic activity of ear epidermis incubated with fraction S_2 from cases 5 and 6 was proved to be remarkably high in comparison to fractions S_0, S_1 and S_3. A similar tendency was recognizable in frac-

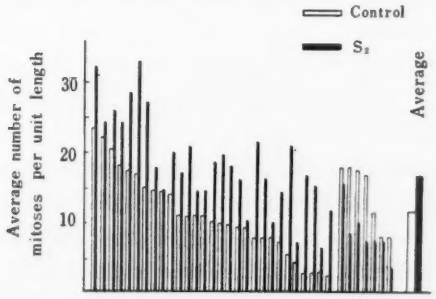


Fig. 5. The effect of the S_2-fraction prepared from a teratocarcinoma of testis (case 5). The average number of mitoses arrested by colchicine in unit length (1cm) of sections of epidermis 7μ thick incubated for 4 hrs. at 38°C in the culture medium. Each pair of histograms shows the effect on an individual ear of a mouse.

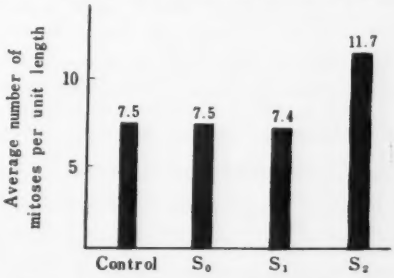
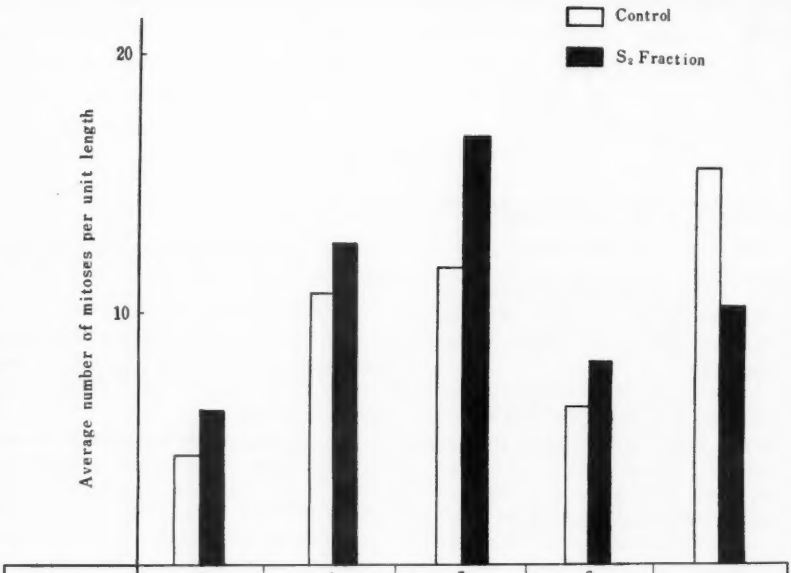


Fig. 6. The effect of the ethanol fractions of a retroperitoneal leiomyosarcoma (case 8). The average number of mitoses arrested by colchicine in unit length (1cm) of sections 7μ thick of ear epidermis of 3 mice, incubated for 4 hrs. at 38°C in the culture medium.



Case	7 Fibrosarcoma of the abdominal wall	8 Leiomyosarcoma of retroperitoneum	5 Teratocarcinoma of testis	6 Seminoma	Normal bovine testis
No. of exp.	33	27	36	18	5
t	0.85	1.40	5.83	1.79	4.99
p	0.4	0.2	0.001	0.1	0.01

t : the value of t−test
p : the level of t−test

Fig. 7. The effect of S_2-fractions prepared from several malignant tumors. The average number of mitoses arrested by colchicine in unit length (1cm) of sections of ear epidermis 7μ thick incubated for 4 hrs. at 38°C in the culture medium. Each value is the average of several experiments.

tion S_2 from case 7 (fibrosarcoma of the abdominal wall) and 8 (retroperitoneal leiomyosarcoma). However in these last cases the difference from the control (fractions S_0, S_1, and S_3) was less significant statistically. Fraction S_2 as well as all other fractions from normal bovine testis, were found to be rather inhibitory to the mitosis.

Discussion

Annau *et al.*[1] reported an increased appearance of mitotic figures in liver of mice transplanted with Ehrlich's ascites tumor. Aizawa[2] in our clinic could also confirm an increased appearance of mitotic figures in liver of the gastric cancer patients in comparison with that of the ulcer patients. Lettré and his collaborators[7] found an

increased mitotic index in cells of Ehrlich's ascites tumor subsequent to intraperitoneal injection of a homogenate of the same tumor. According to them,[8),9),17)] the intraperitoneal injection of the nuclear fragments and mitochondrial and microsomal fractions of the same tumor were equally effective, while injection of the supernatant was ineffective. The effective fraction could be inactivated by heating for 15 min. at 100°C. They therefore concluded that there was a mitosis promoting substance in sedimentable fractions of the homogenate. On the contrary Malmgren,[10)] who confirmed the increased appearance of mitotic figures in liver of mice bearing mammary carcinomas, injected a homogenate of the breast cancer of mice or the saline extract of the same tumor subcutaneously into normal mice, and found an increased mitotic index in the liver. According to him, the injection of mitochondrial and microsomal fractions was ineffective. Recently Ogawa *et al.*[13)] observed an increased mitosis in liver of rats in which a rat fibrosarcoma had been transplanted. They[14),15)] reported, too, that the injection of the extract of infantile rat tissue, or of the human pregnant serum had the same effect on cells of Yoshida sarcoma *in vivo*.

All these were *in vivo* experiments and concerned briefly with calculation of the mitotic figures after various periods of time following the injections. Not only the fraction, but also the period in which the mitotic figures were most frequently found, differs greatly. The peak was observed in Ogawa's experiment on the 4th day and 2-3 weeks after transplantation or injection, in Malmgren's experiment 48 hours after injection and in Lettré's experiment 8 hours after injection.

In *in vivo* experiments, even with pure strains, the difference between individual animals is by no means negligible, so that analysis of data is often difficult. The *in vitro* experiments overcome this defect and facilitate an objective comparison. It is self-evident, both in *in vivo* and *in vitro* experiments, that the increased mitotic index following administration of certain substances, can by no means be directly ascribed to the mitosis promoting activity of these substances. For, those substances, which retard mitosis, can equally well result in an increased number of mitotic figures in the preparation. Bullough's method,[3),4),5),6)] originally devised for the examination of the influence of hormons upon the mitosis of the epithelium, is quite ingenious in arresting mitosis at the metaphase by 4 hour's incubation of a piece of epidermis with the materials to be tested. With this method the mitoses which took place in a certain area of epidermis can be numerically calculated.

We succeeded in distinguishing from a saline extract of teratocarcinoma and seminoma of man a substance (or substances) which promotes mitosis *in vitro* in the epidermis of normal mice. It was found in the 6th (or 5th) fraction on a starch colum chromatogram and was precipitated from the above human testicular tumors by 30–70% ethanol. The same ethanol fraction of two rapidly growing sarcoma was also effective, while the analogous fraction of normal bovine testis was ineffective.

We would like to denominate this substance "oncotrephin*". As to the nature of oncotrephin further investigations are in progress. Since the effecting modes of the substances of previous investigators differ greatly not only from each other but also from ours, their identification with ours seems unlikely.

Conclusion

A study was made with certain human malignancies to detect a substance (or substances), which promotes *in vitro* the mitosis of ear epidermis of normal mice.

The mitosis promoting effect was confirmed in the 6th (or 5th) fraction among 12 isolated by starch column chromatography from 4 human malignant testicular tumors.

Similar substance was present in the fraction precipitated with 30–70% ethanol from 2 cases of malignant testicular tumors.

The authors wish to thank deeply Dr. Y. Aoki, Dr. M. Oziri and Dr. T. Utunomiya for their helpful collaboration.

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*'ὄγκος (tumor), τρόφειν (to make grow).

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FURTHER STUDIES ON A MITOSIS PROMOTING SUBSTANCE (ONCOTREPHIN) IN HUMAN MALIGNANT TUMORS: ITS EFFECT ON STRAIN L CELLS*

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In the previous report[6] Kuru, Kôsaki, Matuda and Hukui demonstrated the existence of a substance in certain human malignancies which promotes the mitosis of mouse epidermis *in vitro*. This substance is present in the 6 th (or 5 th) fraction of crude extract of these tumors on a starch column chromatogram and can be precipitated with 30-70% ethanol. The present study is concerned with the influence of the same substance on the growth of strain L cells.

MATERIAL AND METHODS

Two malignant testicular tumors and one spindle cell sarcoma were used. The testicular tumors were the 5 th and 6 th case described in the previous report. The material used for this study is briefly summarized in Table 1.

Table 1. Tumors used for preparations

Case	Age	Sex	Clinical course	Source	Location	Histological diagnosis	Weight (g)
1	25	♂	Cryptorchidism (l.), operated under diagnosis of ileus. No metastasis.	Operation	L. testis in abdominal cavity	Seminoma	360
2	1	♂	Since 6 mo., l. testicular tumor. No metastasis.	Operation	L. testis	Terato-carcinoma	40
3	58	♂	Ca. 5 yrs. ago apple size l. scapular tumor extirpated. Ca. 2 yrs. ago recurred, child head size tumor extirpated. Man head size tumor recurred again.	Operation	L. scapular region	Spindle cell sarcoma	1500

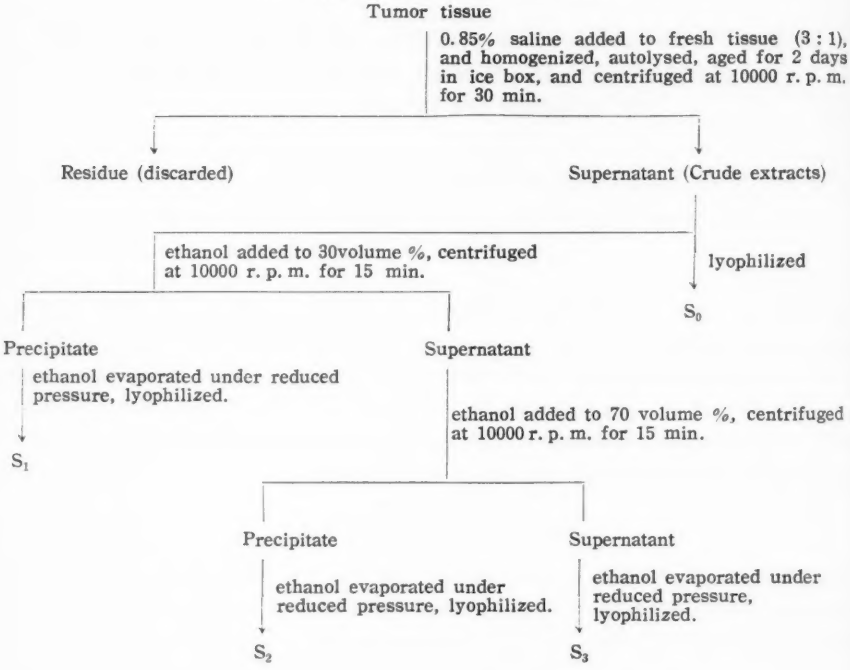
Case 1 Correspond to the 6 th case in the previous report.

Case 2 Correspond to the 5 th case in the previous report.

The crude extract S_0 and fractions S_1, S_2, S_3 were prepared as described in the previous report. The procedure used is indicated briefly in Table 2.

* A part of the research expenses was paid by the Scientific Research Fund of the Ministry of Education.

Table 2. Preparation of the mitosis promoting substances from rapidly growing tumors of man.



Tissue culture strain L cells kindly supplied by Dr. H. Katsuta were used.

To estimate accurately the increase in number of cells in culture vessels, the simplified replicate tissue culture method[4),5)] was employed. As culture vessels, short test tubes graduated at 0.5 and 1.0 ml were used. A half ml of the cell suspension in Hank's saline was transferred into each of these tubes and then 1 ml of nutrient fluid was added. In most experiments, 1.5 ml of this culture medium consisted of 5% bovine serum and 95% Hank's saline containing 0.5% lactalbumin hydrolysate and 0.1% yeast extract. In the experimental group, the test material was added to the culture medium at various final concentrations such as 1 mg/ml or 0.2 mg/ml. In the control experiment no test material was added. The pH of the culture medium was adjusted to 7.6. Tubes were sealed with double rubber stoppers and were placed at an angle of 5° and kept at 37°C. The whole culture medium was renewed every other day after decanting the liquid from the tubes.

The cultivation was usually carried out for a week, and three or four (usually three) tubes were used for counting on the 2nd, 4th and 7th days. The zero reference day indicates the day of inoculation. For the isolation of cell nuclei, 0.1 M

citric acid containing 0.02% crystal violet, was added to eight times its volume of cell suspension. Tubes were kept unshaken at room temperature for 2 hrs., and then were centrifuged at 1500 r. p. m. for 10 minutes after mixing well with a pipette. Following the graduation of 1 ml of the glass wall of the tube, the supernatant was removed to a total volume of 1 ml. After mixing with a pipette, the nuclei in suspension were counted in a hemocytometer.

Results

The effect on the growth of strain L cells of fractions S_0, S_1, S_2 and S_3 from case

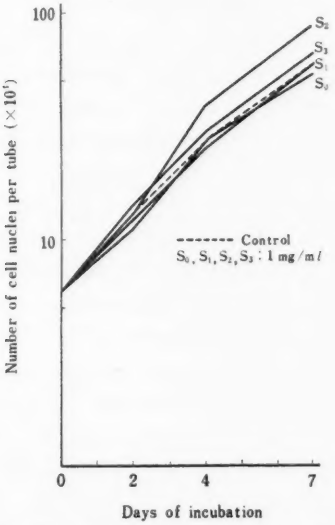


Fig. 1. Effect of fractions S_0, S_1, S_2 and S_3 prepared from a seminoma (Case 1) on proliferation of Strain L cells.

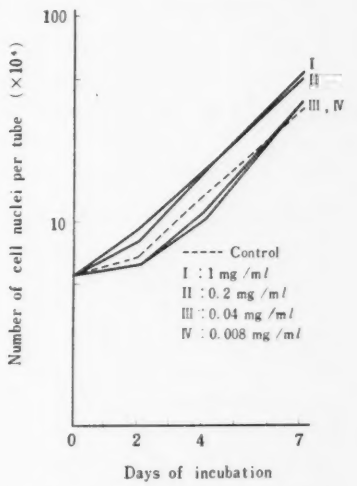


Fig. 2. Effect of different concentrations of fraction S_2 from a seminoma (Case 1) on proliferation of Strain L cells.

1 is shown in Fig. 1. On the 4 th day of culture the effect of fraction S_2, at a concentration of 0.5 mg/ml, is already recognizable and the effect continues to the 7 th day. It produces an increase by 35% in the number of cells over the control. On the contrary fractions S_0, S_1 and S_3 have no effect. However even with fraction S_2 no effect was observed if the concentration is increased to 5 mg/ml or decreased below 0.04 mg/ml (Figs. 2 and 3). If fraction S_2 was added at a concentration of between 1.0 mg/ml and 0.2 mg/ml an increase of 30-40% in the cell count over the control value was constantly observed (Fig. 3). As in the previous experiments, addition of fraction S_2 from normal bovine testis had no effect, even at a concentra-

tion of 1.0-0.2 mg/ml (Fig. 3).

A similar result was obtained by addition to the culture medium of fraction S_2 at a concentration of 1.0-0.2 mg/ml from another testicular tumor (case 2). In this case a 25% increase in cell count was observed in comparison with the control experiment (Fig. 4) or experiments with other fractions. A much greater effect resulted from the addition of fraction S_2 from a spindle cell sarcoma (case 3). In this case at a concentration of 1.0-0.2 mg/ml, a 70% increase in cell number was recorded in comparison to the control (Fig. 5). Other fractions again proved ineffective even at the same concentration.

Discussion

Glinos *et al.*[3] and Friedrich-Freksa *et al.*[2] found a substance which promotes mitosis in liver in blood from partially hepatectomized rats. Accord-

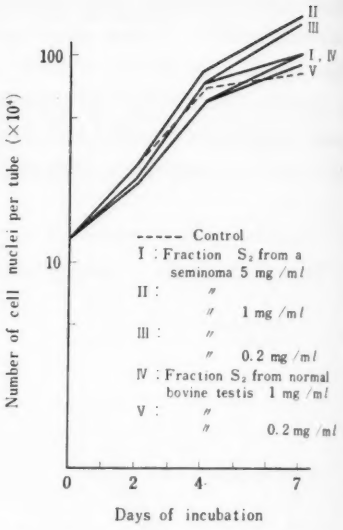


Fig. 3. Effect of fraction S_2 from seminoma (Case 1) and from normal bovine testis on proliferation of Strain L cells.

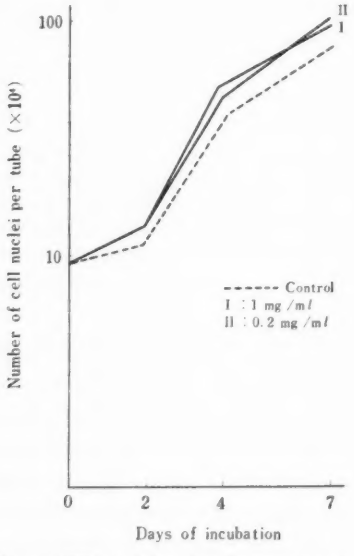


Fig. 4. Effect of fraction S_2 from a teratocarcinoma (Case 2) on proliferation of Strain L cells.

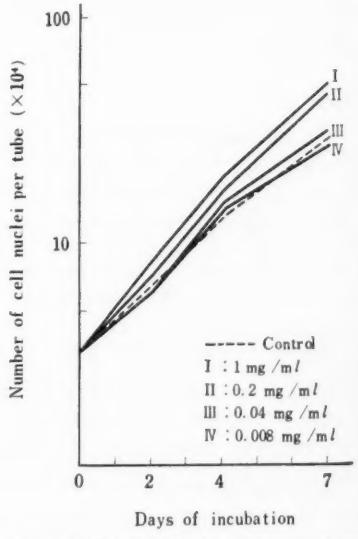


Fig. 5. Effect of different concentrations of fraction S_2 from a spindle cell sarcoma (Case 3) on proliferation of Strain L cells.

ing to the latter author, this substance is present in the serum protein fraction and influences the liver organ-specifically. Recently Ogawa *et al.*[7] succeeded in demonstrating, by tissue culture experiments, an analogous substance in the serum of rats from which one kidney had been removed 2 days previously. This substance is also organ-specific and selectively promotes mitosis of kidney cells in tissue culture. According to these workers this substance is non-dialysable, and is still effective after 30 minutes' heating at 56°C, though it can be inactivated by heating for 20 min. at 100°C. This substance is said to be organ-specific but not species-specific. On the 2nd day after removal of one kidney a compensatory hyperplasia of the other kidney had already commenced. Consequently, at this stage an abundant mitosis can be observed in the latter organ. Generally in those organs or tissues in which abundant mitosis takes place, the existence of a certain substance which promotes cell division humorally can be assumed. Indeed, Teir and his co-workers[1] succeeded in distinguishing, in a homogenate of regenerating liver, in which increased mitosis is recognizable, a substance which promotes the mitosis of liver cells *in vivo*.

It is therefore quite reasonable to assume a mitosis promoting substance in malignancies in which there is much mitosis. Three of the present authors and Hukui[6] elucidated the existence of such a substance in 6 cases of testicular tumors, in which especially an accelerated growth is noted. This substance was precipitated from a crude extract by 30–70% ethanol and was proved to promote the mitosis of normal rat epidermis *in vitro*. Ulloa-Gregori *et al.*[8] had already confirmed the growth-promoting effect of human cancerous ascitic fluid on cultured human epidermis cells. According to the latter workers, there is a considerable difference in growth promotion between cancerous ascites or human umbilical cord serum and ascites resulting from hepatic cirrhosis or human serum. In their experiments, however, the distinction of the growth promoting effect was made by comparing the areas of outgrowth, utilizing a projection technique. In our present experiments strain L cells were employed. In this strain, originally isolated from mice fibroblasts and established by Earl, not only does the growth rate have a constant graphical representation, but also in calculating the nuclei, a numerical estimation of growth promotion is possible. Another advantage of using this strain is the culture medium. For cultivation of this strain it is unnecessary to add an extract of chick embryo, which itself is a source of growth promoting factors. We confirmed in this experiment that fraction S_2 of malignant testicular tumors and a spindle cell sarcoma contains a substance which, in optimal concentrations, promotes the growth of strain L cells. The same fraction from normal bovine testis had no effect. These results correspond well with those of previous investigations, in which the mitosis promoting effect was tested with normal rat epidermis *in vitro*.

Conclusion

A fraction from human testicular tumors and spindle cell sarcoma, which is precipitated with 30-70% ethanol from a crude extract, when added to the medium, produces a 25-70% increase in cell number of strain L cells on the 7 th day of tissue culture. This result is in accordance with the previously reported results using Bullough's method.

The authors wish to thank Dr. Y. Aoki and Miss E. Yosioka sincerely for their helpful collaboration.

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[GANN, 50, 127~141 ; June, 1959]

CYTOLOGICAL STUDIES OF TUMORS, XXV. A TRANSPLANTABLE RAT ASCITES HEPATOMA PRODUCED BY P-DIMETHYLAMINOAZOBENZENE[1]

(Plates IV and V)

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Ascites tumors of rats and mice have supplied useful material for the study of chromosomes of tumors, and observations in the recent several years have provided valuable cytological data for the understanding of the nature and development of tumors. The cytological studies of transplantable rat ascites tumors were reviewed by Makino (1957). His major conclusion was that each tumor studied is characterized by a stem-line (or-lines) of tumor cells which are primary contributors to the neoplastic growth and which maintain the genetic pattern of each tumor. During the last several years the author has been engaging in histological and cytological observations on hepatocarcinogenesis by the application of azo-compounds such as o-aminoazotoluene and p-dimethylaminoazobenzene (Tanaka 1952, Tanaka and Kanò 1954). In order to provide material for advantageous study of the chromosomes, a hepatoma induced by the agent was transformed into an ascites form. Previously it has been reported that solid hepatoma tissues of rats were able to produce freely suspended cells in the peritoneal cavity (Yoshida, Sato and Aruji, 1951, Tanaka and Kanò, 1952, Yoshida, 1956, Sato, Belkin and Essner, 1956).

The present paper describes the procedure of experimental hepatocarcinogenesis in rats, the general features of the ascites hepatoma produced, and the behaviour of hepatoma cells following treatment with some enzymes.

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Materials and Methods

The primary hepatoma was induced in a male Buffalo rat by applications of p-

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dimethylaminoazobenzene (DAB). The animals used in experiment were about four months of age and weighed 150 to 180 grams each. Fifty rats were daily administered for a period of 210 days with feeding a carcinogenic diet according to Harada *et al.* (1937) which contained 0.06 per cent of DAB. They were then fed with Makino Laboratory chow pellets (Sato and Okumura 1957) without containing carcinogene. Thirty-two animals developed single or multiple tumors in livers 9 to 14 months after the beginning of application of carcinogenic diet. A primary solid hepatoma induced by 317 days after the beginning of administration was converted into an ascites form; with it the present study deals. Several stocks of rats,[1] Buffalo, Wister/Ma, Long-Evans, Fischer and Albany, were used for the serial transfers of tumor.

Tumor tissues were fixed in Orth's fluid. Sections 8 microns thick were prepared in the usual way and stained with haematoxylin and eosin. Microscopic observations of the tumor ascites were made on temporary preparations stained with acetic orcein. For the observation of the chromosomes, Sudan Black B squash technique was adopted advantageously (Tanaka and Kanô 1957). Succinic dehydrogenase activity of tumor cells was tested according to Hirono's method (1957).

Results

I. Establishment of an ascites hepatoma.

The primary hepatoma was induced in a male Buffalo rat by 317 days after beginning of DAB administration. It consisted of a white primary nodule in the hepatic tissue. By autopsy, some nodular metastases were observed on the intraphragmatic surface, without any neoplastic exudation in the peritoneal cavity. Microscopically, the primary tumor resembled in general appearance the hepatocellular carcinoma induced by DAB feeding (Figs. 1-2). The cells were generally of large size, and the basophilia of their cytoplasm was intensely and diffusely dispersed. Some areas consisting of greatly vacuolated cells were found within the tumor. The binucleated cells and cells with large, irregular bizarre nuclei were frequent. Nucleoli, generally one or two in number, are present in the nucleus of each cell. Mitotic figures, including many of abnormal characters, were abundant.

A suspension of cells from the primary tumor was prepared by macerating the solid, non-necrotic tissue in Tyrode's solution with the aid of a small glass homoginizer. Using a wide bore needle (# 15), 0.5 ml. of the suspension was inoculated intraperitoneally into each of five Bufiallo rats. In all cases, superficial observations showed the occurrence of several small loosely adherent nodules in the peritoneal

1) The author is grateful to Dr. George Gay of the National Institute of Health, Bethesda 14, Md., U.S.A., for geneously supplying to Makino's Laboratory the Buffalo, Fischer, Albany and Long-Evans strain rats. He wishes also to thank Dr. Eugene Roberts, City of Hope Medical Center, Duarte, California, U.S.A., for arranging the shipment of these rats to Japan.

cavity by the 20th to the 27th day after inoulation. It is evident that the epithelial nature of the hepatoma tissue growing in masses persists even after the inoculation into the peritoneal cavity. The tumor cells forming hepatoma-cell islands were occasionally in process of division. There is a possibility that tumor cells undergoing division are capable of proliferation when they are transferred into the peritoneal cavity. With this view in mind the inoculation experiments were carried out further as follows:

A saline solution, containing minced hepatoma tissue harvested from one of the rats, positive to transplantation was inoculated intraperitoneally into four rats. Abdominal fluid obtained from the above rats five days after inoculation was hemorrhagic and contained tumor cells as well as inflammatory cells. Tumor cells, some being in mitosis, grouped in clusters or formed islands. From one of the above rats, ascites fluid containing the tumor-cell islands was drawn on the 17th day after inoculation, and then inoculated intraperitoneally into in three rats. All rats thus treated developed ascites fluid which was similar in nature to the transplant and contained a large number of hepatoma-cell islands of varying sizes and shapes. After this generation, the ascites hepatoma showed a rapid growth in the peritoneal cavity of rats.

Serial transfers of the ascites hepatoma have been carried through 137 passages (January 1959). The author proposes to designate this tumor by the name "ascites hepatoma II" in the following.

II. General characteristics of the ascites hepatoma II.

Several strains of transplantable ascites hepatomas in rats and mice have previously been reported (Sasaki 1958, Sato et al. 1956, Tanaka and Kano 1952, Yoshida 1956, Yoshida *et al.* 1951). The characteristics of the present ascites hepatoma are described as follow:

1) Growth pattern of the ascites hepatoma II: The ascites fluid, when examined under the microscope, is seen to contain typical hepatoma-cell islands and a few freely suspended tumor cells. In each tumor-animal there are usually 15–20 ml. of ascites fluid of milky or hemorrhagic viscous nature, at 17 to 20 days after inoculation. Observations of cells made at various times in a transfer generation show that hepatoma cells are approximately 90 per cent in occurrence while the remainder are leukocytes, macrophages and connective tissue cells. The above proportion has remained unchanged throughout 137 transfer generations so far examined.

During early transfer generations, the tumor growth was rather slow as compared with that of other ascites hepatomas previously reported. Death of host animals usually occurred between the 30th and the 37th day or more later, the extreme life span being over 50 days. The transplantability was 100 per cent for Buffalo rats, while remarkable invasive tumor growth in the visceral organs occurred in all animals so far employed: tumor tissues first extend their small nodules on the surface of the

peritoneal organs and then infiltrate into the interior of organs. The tissues in which invasion was observed are omentum, retroperitoneum, diaphragma and portal areas. In the early transfer generations most hepatoma-cell islands were composed of 50-80 tumor cells, there being some islands which contain over 100 cells.

With the repetition of transfer generations, the tumor showed a gradual change in nature. From the 24th generation onward, the tumors appeared to grow rather rapidly, with death of host occurring between the 16th and the 20th day. The invasive tumor growth in the peritoneal organs was less widespread than in the early transfer generations as noted above, tending to be confined within the omentum. Along with the alteration of growth characteristics, there occurred a decrease in diameter of hepatoma-cell islands. By the 30th transfer generation, each tumor island was composed of 10 to 40 tumor cells, with no island having more than 100 cells. A similar feature was found by Sasaki (1958) to occur in ascites hepatoma 17. Figure 4 shows a typical tumor ascites in the 30th transfer generation.

2) Hepatoma-cell island: The ascites fluid examined by bright phase microcopy shortly after removal showed a predominant occurrence of hepatoma cell islands which were composed of several tumor cells. The border of each island was generally smooth, though there were sometimes observable pseudopodial projections from the cells (Fig. 5). The bonds that hold each cell of the island together vary in their tightness. The cytoplasm of cells forming each of the islands contained a number of shiny particles, being apparently lipoidal in nature as they were readily stained with scharlach red. Sometimes, the cells showed cytoplasm which contained minute granules negative to the fat stain. The particles showed active Brownian movement. The nucleus was round or oval-shaped and contained one or more nucleoli round or elongated in shape. In addition, there were in the ascites fluid hepatoma-cell islands in process of disintegration, particularly in the later stages of the tumor-animal's life span (Fig. 6). The cells forming islands contained many vacuoles occupying much of the cellular space.

Succinic dehydrogenase activity of mitochondrial enzyme was examined in the ascites hepatoma II. This was readily done with neotetrasolium. The results showed that the precipitated formazan granules are localized in the cytoplasm, while the nuleus are free from the precipitation. The exclusive localization of formazan in the mitochindria in several cells is shown in Figure 7. Comparative microscopic examinations made of cells with and without succinic dehydrogenase staining revealed that the hepatoma cell islands showing moderate enzyme activity were of morphologically normal appearance. Each cell forming the islands was smooth in outline and contained an oval nucleus with one or two nucleoli, and the bonds that hold each cell of the island together were very tight. Cells containing less formazan precipitation differ in their morphology from the former: they were large in size and more or less vacuo-

lized, their cellular adhesiveness being rather weak. Their nuclei were of a circular shape and had mostly lost their basophilia. Based on the above evidence it is highly probable that degenerative processes had taken place in them.

3) Transplantability of the ascites hepatoma II in various stocks of rats: Table 1 summarizes the results of observations on transplantability of the ascites hepatoma II in rats from five different stocks. It is obvious from the table that Buffalo,

Table 1. Transplantability of the ascites hepatoma-II in five different stocks of rats.

Rats stocks	Number of animals transplanted	Number of tumor animals died	Per cent of transplantability
Buffalo	112	112	100
Wistar/Ma	104	88	84.6
Long-Evans	32	25	76.5
Fischer	20	4	0
Albany	21	14	66.6

Wistar / Ma and Long-Evans rats are highly sensive to the tumor, showing transplantability at 100 per cent for Buffalo, 84.6 per cent for Wister / Ma, 76.5 per cent for Long-Evans. After inoculation of the tumor in these stocks, the tumor cells proliferate rapidly, and within two to five days after inoculation the tumor cells are in a state of pure culture with the accumulation of a milky neoplastic exudate in the peritoneal cavity. After 12 to 17 days, the ascites fluid become hemorrhagic and thinner in nature and increased in quantity. Then the death of the host animals follows.

Rats which died of the present tumor were comparatively less in number in the Albany stock being 66.6 per cent. The pattern of growth or regression of the tumor in rats of this stock is similar to that observed in other stock rats.

A few rats of Wistar / Ma. Long-Evans and Albany, showed spontaneous post-inoulation regression of the tumor. In these cases the proliferation of tumor cells ceased within 7 to 12 days after inoculation, with subsequent degeneration of tumor: the hepatoma cells showed swelling and loss of affinity to stain, forming numerous vacuoles in cytoplasm. Meanwhile they underwent karyolysis and cytolysis and finally disappeared.

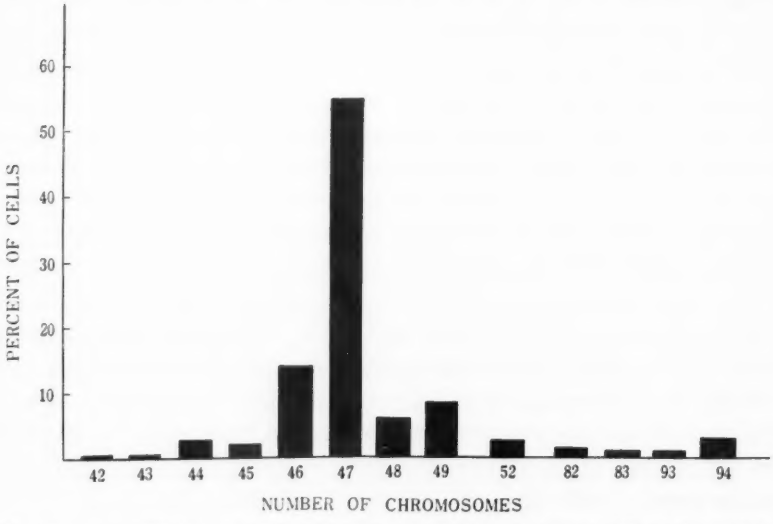
In the transplant experiments with the Fischer rats, no animals died of disease. The Fischer rats appear to be nonsusceptible to the present ascites hepatoma II.

4) Morphological changes of the hepatoma-cell islands in a transplant generation: In samples of the ascites fluid taken periodically after inoculation, the morphological changes occurring in the hepatoma-cell islands were observed. Many of the cells forming the islands, especially comparatively large islands, began to disintegrate in a new host soon after inoculation, showing blisters on the surface of the tumor cells. This followed a formation of a hepatoma-cell island of small size, along with free

tumor cells. They increased in number considerably for one to two days after inoculation. The cells, particularly in the periphery of each island, divided by active mitosis. Consequently, the island became irregular in shape often becoming a claster. The mitotic rate of tumor cells in each island showed the maximum in the middle part of the life span.

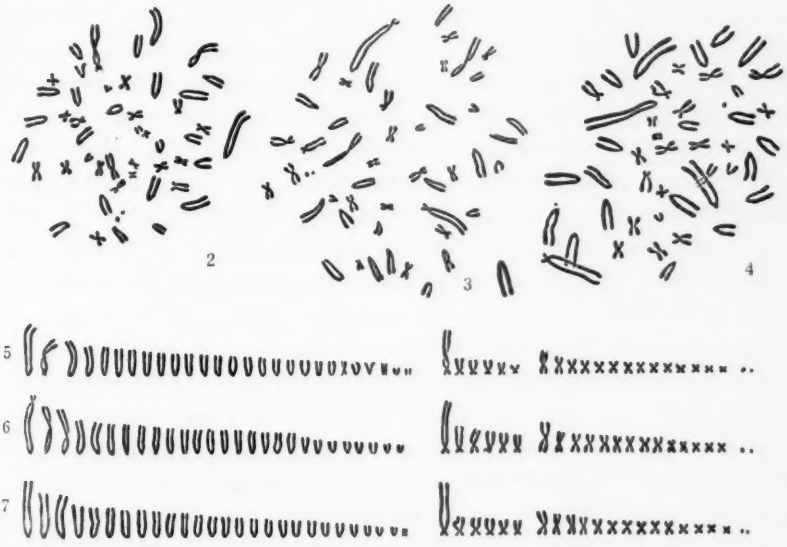
With the passage of time, the disintegration of hepatoma cells became remarkable with the production of a considerable number of small islands and free tumor cells, the latter being much fewer in number than the former. Remarkable is the occurrence of two types of free tumor cells: one type consisting of large spherical cells with many cytoplasmic granules, is common in occurrence; the other contains elongated cells with densly stained cytoplasmic processes for amoeboid movement. In addition there were freely floating tumor cells showing degenerative changes which are also found especially in the first type of cells. Occasionally, these free tumor cells show adhesion with each other into a bead-like form or a small cluster which grew into islands of various sizes. The above findings suggest that the tumor cells forming the islands have remained is a condition favorable for living, while in an isolated condition free from the island the cells may be unsuited to live. It seems probable that in an island the cells are living in a manner like symbiosis.

5) Chromosome constitution of the ascites hepatoma II.: In samples derived from the 1st to 5th transfer generation, the chromosome number of the present tumor showed a fluctuation ranging from 32 to 54. Thereafter, the frequent variation of



Text-figure 1. Histogram of the chromosome numbers in tumor cells of the ascites hepatoma II. This analysis is based on 116 critically countable metaphase plates.

chromosome numbers fell within a rather narrow range with the appearance of modal numbers. The samples at the 47th generation showed a range of variation from 44 to 49. The cells showing the modal numbers within this range were 79.3 per cent. Text-figure 1 is a histogram indicating the chromosome-number distribution based on 116 cells taken at the 47th transfer generation: the chromosome number distribution is characterized by a marked peak at 47 chromosomes (52.5%). An idiogram analysis was made on the basis of fifteen clear metaphse plates containing 47 chromosomes. Figures 8 to 10 and text-figurs 2 to 4 are examples of metaphase chromosomes, while text-figures 5 to 7 show serial alignments of chromosomes in descending order of size in each telocentric and metacentric group. The individual chromosome varies somewhat in length from plate to plate due probably to the constraction of chromosomes. Every idiogram is characterized by containing constantly 27 chromosomes of rod-type and 20 J- or V-shaped elements varying in size. The evidence here presented strongly emphsizes that there exist in the present tumor the most frequently occurring tumor cells which posses characteristic chromosome-number mode along with a particular chromosome pattern. In reference to the stem-cell concept established by the investigations of various types of transplant ascites tumors of rats and mice (Makino 1957, a, b, c, 1958, Hauchka and Levan 1958, Levan 1957) the tumor cells characterized by



Text-figures 2–4. Metaphase plates of the tumor cells having 47 chromosomes. Camera-lucida darwing.

Text-figures 5–7. Serial alignment of chromosomes of the ascites hepatoma II, in approximate order of size.

the particular chromosome-number mode and pattern form a stem-cell lineage, the number of which contributes to the growth of this tumor by proliferation. The morphology of the chromosomes in detail will be reported elsewhere in the near future.

6) Behaviour of tumor cells in subcutaneous or intermuscular injections:

The ascites fluid of the ascites hepatoma II was injected subcutaneously, or intermuscularly, and behaviour of tumor cells was studied. The subcutaneous injections resulted in the formation of solid tumors at the site of injection; 37 serial transfers were maintained by injecting suspensions of the solid tumors produced. The intermuscular injections were continued for 25 or more generations. No observable differences of the solid growth of the tumor were detected either in subcutaneous or intermuscular injections. Detailed description of subcutaneous growth of the tumor follow.

Five Buffalo rats received subcutaneous injections in the axillary region with 0.5ml. of the ascites fluid, removed on the 6th day from a rat of the 17th passage. Three of them showed a single progressively developing tumor at the site of the injection. Sample of tumor taken from one of the positive rats was used for the next transplantation. After the 4th generation, the tumor growth was stabilized. The tumor grew rapidly and extensively as solid, but soft masses. Sixty-seven out of seventy-two rats which received transplantations showed a remarkable solide tumor in each case. By the end of the first week the tumor was observed as a small firm nodule, about 0.5cm in diameter. The subsequent growth was rapid. The outer dimension of a tumor observed in a rat 16 days after inoculation was 4.7×5.4cm. In some rats, the central zone of the tumor tissue consisted of a yellow substance of necrotic nature. Microscopical features of the tumor are shown in Figure 3. In more advance stages, the tumor showed varying sizes of the necrotic area. They were commonly multilocular in structure with oozed fluid and blood on dissection. None of the rats showed tumor metastases, but in two rats, the tumor had eroded through the peritoneal wall with an accumulation of ascites. The latter two rats died on the 24th and 29th day respectively: they were the only two examples that were recorded in the entire series of experimental rats.

Tumor suspensions of the subcutaneous passage at the 10th, 20th and 30th generations were injected intraperitoneally. By the 10th and the 20th passage, all of injected rats, five each, were autopsied on the 7th day of inoulation. They all showed abdominal distensions with a copious volume of ascites fluid containing a number of hepatoma-cell islands. Suspensions of the 30th the passage, however, were not easily convertible into an ascites form: in some animals, hepatoma-cell islands were not detectable in the peritoneal fluid at all, while some others showed a few hepatoma-cell islands during the first few days after inoculation, which completely disappeared in a short time. It is remarkable that all animals died with solid tumors which developed in the peritoneal cavity or adhered to abdominal wall. Such solid

tumors produced highly hemorrhagic exudations which occasionally contained a few hepatoma-cell islands, the proportion of which was very low being 2 to 5 per cent. The tumor tissues were removed asceptically and suspended in the peritoneal fluid of fresh animals by a routine method. With continuation of such a treatment, an ascites tumor line was again obtained after the 6th generation. A comparison of the morphological structure and the chromosome pattern of the ascites tumor thus induced with those of the original one showed no significant difference between them. No conclusion can be drawn as to the cause of alteration in the convertibility which occurred in the present study. The reports of Klein (1953, 1954) indicate that the ability of a tumor to convert into an ascites form is due in part to a rapid growth rate and / or high degree of anaplasia, or what is perhaps more important, to a process of selection of cells possessing a predilection for growth in the ascites form. Klein also stated that the selective advantage of the tumor cells can be increased during the process of gradual conversion. These conceptions may be applicable to the interpretation of the present experimental results.

III. Behaviour of the hepatoma cells following enzymatic digestion: The following experiments were undertaken to accelerate the disintegration of the hepatoma-cell islands by treating them with certain enzymes. The hope was to be able to ascertain whether or not the single cells free from islands are capable of proliferation in an isolated status.

Several kinds of enzymes were used for experiment. Hydrolytic enzymes such as hyaluronidase, carbohydrase and lipase were not effective, but crude trypsin, crystalline trypsin and crystalline chymotrypsin, dissolved in 0.1M phosphate buffer solution (pH 7.4), caused almost complete digestion of islands. The whole procedure has previously been described in detail (Tanaka 1952); only the essential are given below:

The ascites fluid was incubated for 10 minutes at 38°C in an isotonic saline solution without calcium and magnesium salts. It was then transferred to a 0.5 per cent solution of trypsin or chymotrypsin. After about 15 minutes in this solution, the hepatom-cell islands were almost completely separated: many cells appeared in spherical shape, in some cases, somidetached (Fig. 11). The samples thus treated were thoroughly washed in the phosphate buffer solution by centrifugation and then inoculated intraperitoneally into fresh animals.

Within 2 to 3 hours after inoculation, almost all hepatoma-cell islands became free from islands, though a very few cells, less than one per cent, remained forming islands (Fig. 12). By about 5 hours, free tumor cells protruded blister-like processes, variable in length and shape, from the surface. These protrusions, which often terminated in a knob-like thickening, showed constant movement, similar to the bubbling observed in dividing cells in tissue culture. These changes may probably represent either the reaction of cells to isolation, or a specific response to the perito-

neal fluid. In the course of time some of the discreted cells began to move by means of amoeboid pseudopodia, some being round and blunt, while others are long and tapering (Fig. 18). The tip of the cells showing active amoeboid movement was knob-like in form increasing progressively in size; the cytoplasm became constricted until the cell was in the shape of two spheres, one containing the entire amount of cytoplasm, the other the nucleus, with a cytoplasmic connection between them; the nucleus then migrated into the cytoplasmic sphere. Figures 12 to 16 present a succession of pictures of a cell movement as described above.

When two or more cells come near-by, they contact with their processes and then adher in most cases; meanwhile the cells round up and become incorporated in a cluster. Thus, new islands were formed again. The process is shown in Figures 19 to 20.

The reconstruction of hepatoma-cell islands was initiated during a period from 6 to 8 hours after the inoculation of treated coagulum; the process continued for 15 to 18 hours. About 24 hours after inoculation, over seventy per cent of tumor elements in the peritoneal cavity had taken the form of small hepatoma-cell islands. Their numbers and sizes showed a gradual increase with time. Though the reformation of hepatoma-cell islands seems to take place through aggregation of free tumor cells, other factors such as non-disjunctive cell division or cell division following union of daughter cells may also play an important role, as suggested by Tanaka (1952) and Sato *et al.* (1956). In fact a certain number of free tumor cells began to proliferate about 6 hours after the inoculation of treated coagulum; their daughter cells failed to separate completely and became incorporated in a island. The island then developed into a large hepatoma-cell island through mitotic division. At the present, it is uncertain which factor is in favor of the reformation of the hepatoma-cell island. It is noticeable that with time the percentage of free cells markedly decreased with a nearly equivalent rise in the percentage of tumor-cell aggregations.

A small number of free tumor cells round in shape, however, occurred in the peritoneal cavity at a later stage after inoculation. They apparently had lost their adhesive nature. With the use of a micromanipulator, they were inoculated intraperitoneally into some fresh animals. They formes no islands and finally underwent degeneration. But there was a single case out of 34 trails in which free form hepatoma developed in the peritoneal cavity of a rat. The cells in this case completely lost their ability to grow in contiguity. The ascites hepatoma thus developed was transplantable: each free tumor cell produced free daughter cells. It is evident that some tumor cells showed an accidental change in the property as epithelial origin. This change merits attention, since the increase of virulence of tumor tissue is closely correlated with reduction in, or less of adhesiveness to one another.

Discussion

Several attempts have been made to transfer the malignant tumor of the rat and mouse into an ascites form (cf. Klein 1956). The cells of ascites tumors grow in the peritoneal cavity by repeated multiplications without showing cellular aggregation. In striking contrast to typical ascites tumors, the ascites hepatoma II here under study grow more or less exclusively by multiplication of suspended clusters and clumps of tumor cells; even after extensive disruption of hepatoma-cell islands by certain enzymes, the cells formed islands again. Here the questions arise: (1) why do the hepatoma cells adhere with each other, and (2) do the cells adhere with their naked surface or with certain intercement substances.

The results of the present experiments show that free cells isolated from the tumor-cell islands by means of enzymatic digestion joined rapidly in a peritoneal cavity. Once a small cluster of tumor cells is formed in the peritoneal cavity, the cluster seems to exert a direct influence on the motile cells in its vicinity to construct tumor-cell islands. Probably, the reformation of tumor-cell islands seem to be caused by cellular affinity. Since Roux (1894) first suggested the existence of cytotropic forces of attraction between embryonic cells, various mechanisms have been proposed to interpret the process of cellular aggregation. Loeb (1920) considered that aggulutination was the factor which made isolated cells join into tissue. In sponges, active amoeboid movement is mainly responsible for the aggregation of isolated cells (Galstoff 1925), while in myxomycetes a diffusible chemical agent appeared to be related with formation of plasmodia by the solitary cells (Bonner 1947). Holtfreter (1948), summarizing various aspects of the behaviour of isolated early embryonic amphibian cells, suggested that the cells which happen to come to close to each other cohere together to form a cluster, or repulse each other depending on some physicochemical conditions of the cellular surface. This concept of contiguity effect and tissue affinity is of interest and importance in considering the stability of clusters of tumor cells newly constructed. The factors responsible for the complete cellular aggregation found in some ascites tumors and the tendency toward the cellular adhesion found in closely related neoplasms are of importance in relation to the cause of malignancy in cells. But those factors have remained wholly unknown until the present.

There is another important problem that deals with the existence of certain intercellular cement substance. It has been shown in certain recent studies that the cement substance occurring in association with protein may often be responsible for maintaining the intergrity of certain cellular systems (Moscona and Moscona 1952, Essner *et al.* 1956). In the present experiments, an effective isolation of tumor cells from the cell-islands was induced through the use of trypsin or chymotrypsin. This seems to show that proteins are involved in adhesion. The mechanism involved a

subject for research in the fields of biochemistry and biophysics.

Further interest was focused to the establishment of a free-cell hepatoma line during the course of experiments dealing with enzymatic digestion of the hepatoma-cell islands. In the free-cell hepatoma line established, the tumor cells had lost their ability to grow in contiguity and their growth rate was remarkably high. It seems probable that the cells may themselves impair their capacity to secret certain intercellular cementing substance.

It is questionable whether the free-cell hepatoma line was produced through the experiments with certain chemical agents or was induced by some other causes. There is no example of a simple experimental procedure causing a permanent change in cell property. It is regretable that no detailed chromosome analysis has been made in the free-cell hepatoma line, because the tumor line accidentally failed to grow after the 11th transfer generation. Quite recently, the author has had an opportunity to establish another free-cell hepatoma line which developed spontaneously from the ascites hepatoma II. The chromosomes of this latter free-cell line distinctly differ by comparison with those of the original ascites hepatoma II. On the basis of the findings, the following considerations may be possible, that: 1) certain cells forming a tumor-cell island (or islands) underwent a mutational change which led to the loss of cellular adhesiveness, or 2) the free-cell population was existed, but suppressed its due to certain unfavorable circumstances. The results of the present study are of some significance in connection with the rise and the development of a new tumor line with the alteration of the stem-line chromosomes.

Summary

The present paper describes the procedure of the establishment of the rat hepatoma in an ascites form named ascites hepatoma II, which was induced by administration of p-dimethylaminoazobenzene; notes are presented on the structural features, growth properties, and behavior of hepatoma cells following enzyme treatment. The tumor cells take formation as cell groups called "hepatoma-cell islands". With the passage of serial transfers, the tumor increasingly showed rapid and invasive growth. Along with these changes, there occurred transitions in structural features of the hepatoma-cell islands, in transplantability of the tumor and in the life span of the tumor-bearing animals. Particularly the morphological changes of hepatoma-cell islands in a transplant generation were remarkable.

In early transfer generations, the chromosome number of tumor cells fluctuated within a wide range. In later generations, the variation of the chromosome number became narrow in range, with the modal numbers at 44 to 49. In the recent samplings cells containing 47 chromosomes occurred at the highest frequency forming a stem lineage. The stem-cell of the ascites hepatoma II is characterized by the constant

existence of 27 rod-shaped chromosomes and 20 V- or J-shaped elements varying in size.

The subcutaneous or intermuscular injections of the ascites hepatoma resulted in solid tumor growths. These solid tumors proved transplantable in series, and after the 20th passage they still transfered into an ascites form developing in the peritoneal cavity. Tumor suspensions of the 30th passage did not produce ascites tumor easily.

The application of trypsin or chymotrypsin effectively served to separate cells in many hepatoma-cell islands resulting in the formation of free cell suspension. The free cell population did not continue to proliferate in a free cell state in the peritoneal cavity, but re-formed tumor-cell islands. Some factors responsible for the cellular aggregation were considered. A free cell line was successfully induced in one of 34 experimental trials with enzymatic digestion. Possibilities for the formation of the free cell line were considered.

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Explanation of Plates IV and V

Fig. 1. Photomicrograph of section of the original tumor. Haematoxylin-Eosin. ×100.

Fig. 2. Part of the same section as that shown in Fig. 1. ×600.

Fig. 3. Photomicrograph of section of tumor transplanted subcutaneously. Tenth generation, 15 days after transplantation. Note variability in size of nuclei and nucleolus. HE. ×400.

Fig. 4. General view of the ascites hepatoma II. 47th generation, 4 days after intraperitoneal inoculation. Acetic orcein. ×100.

Fig. 5. Living hepatoma cell island in ascitic fluid removed on the 4th day after intraperitoneal inoculation. ×400, with phase-contrast microscope.

Fig. 6. Degenerative change of hepatoma cell island, showing decreased cell adhesiveness ×300.

Fig. 7. Tumor cell inoculated in succinate neotetrasolium medium. Note the uniform reactivity of the island with remarked precipitation of diformazan in the cytoplasm. ×600.

Figs. 8-9. Photomicrographs of metaphase chromosomes of the tumor. 47 chromosomes. ×1200.

Fig. 10. Separation of tumor cells from hepatoma-cell island following inoculation in 0.5 per cent trypsin for 15 min. at 37°C phosphate buffer. ×100.

Figs. 11-20. Behavior of single tumor cells after intraperitoneal inoculation of discrete hepatoma cells.

Fig. 11. Isolated hepatoma cells three hours after inoulation. ×300.

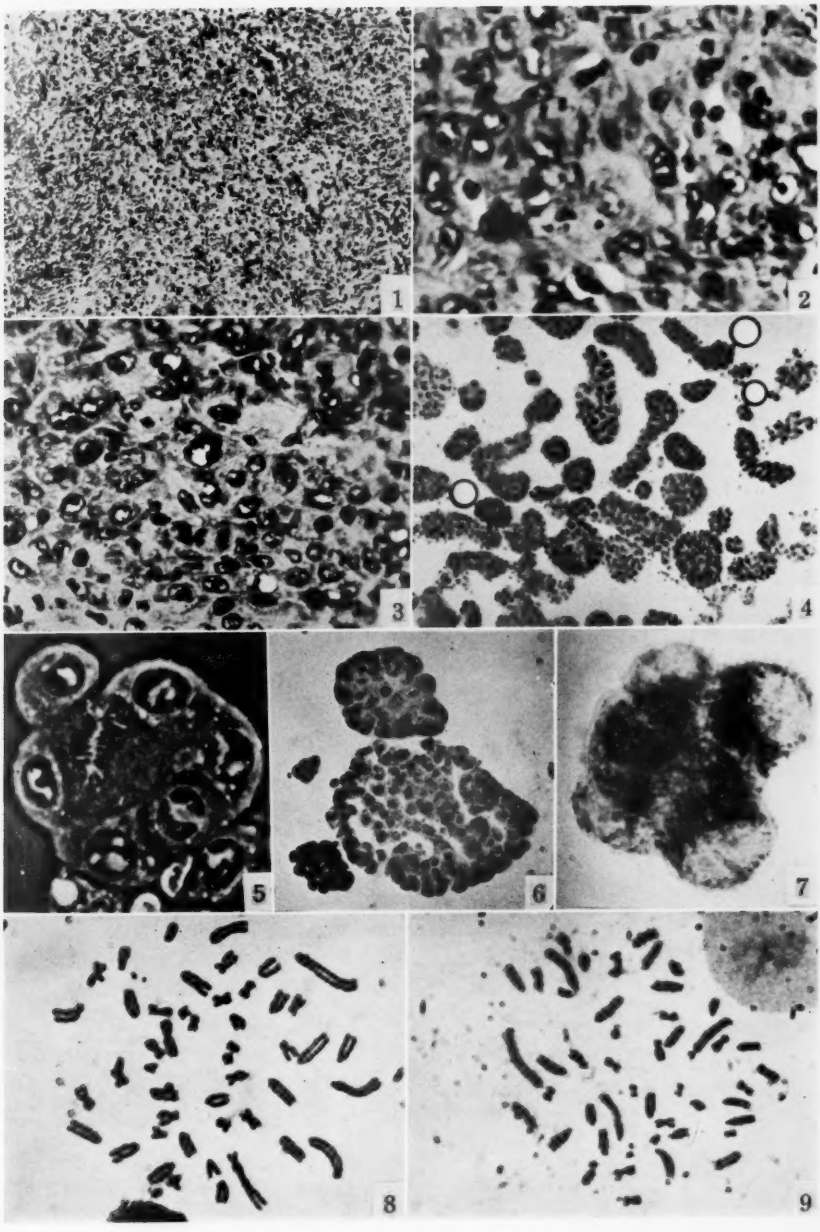
Figs. 12-16. Pseudopodial movement of discrete tumor cells five hours after inoculation. ×800.

Fig. 17. Locomotion of isolated tumor cells in peritoneal fluid, 5 hours after inoculation. ×400.

Fig. 18. Early stage in reorganization of tumor island. Tumor cells shortly after establishing contact, gliding along each other by means of protoplasmic processes. ×400.

Fig. 19. Intermediate stage in reorganization of hepatoma cell island. ×400.

Fig. 20. Final stage in reorganization of tumor cell island. ×400.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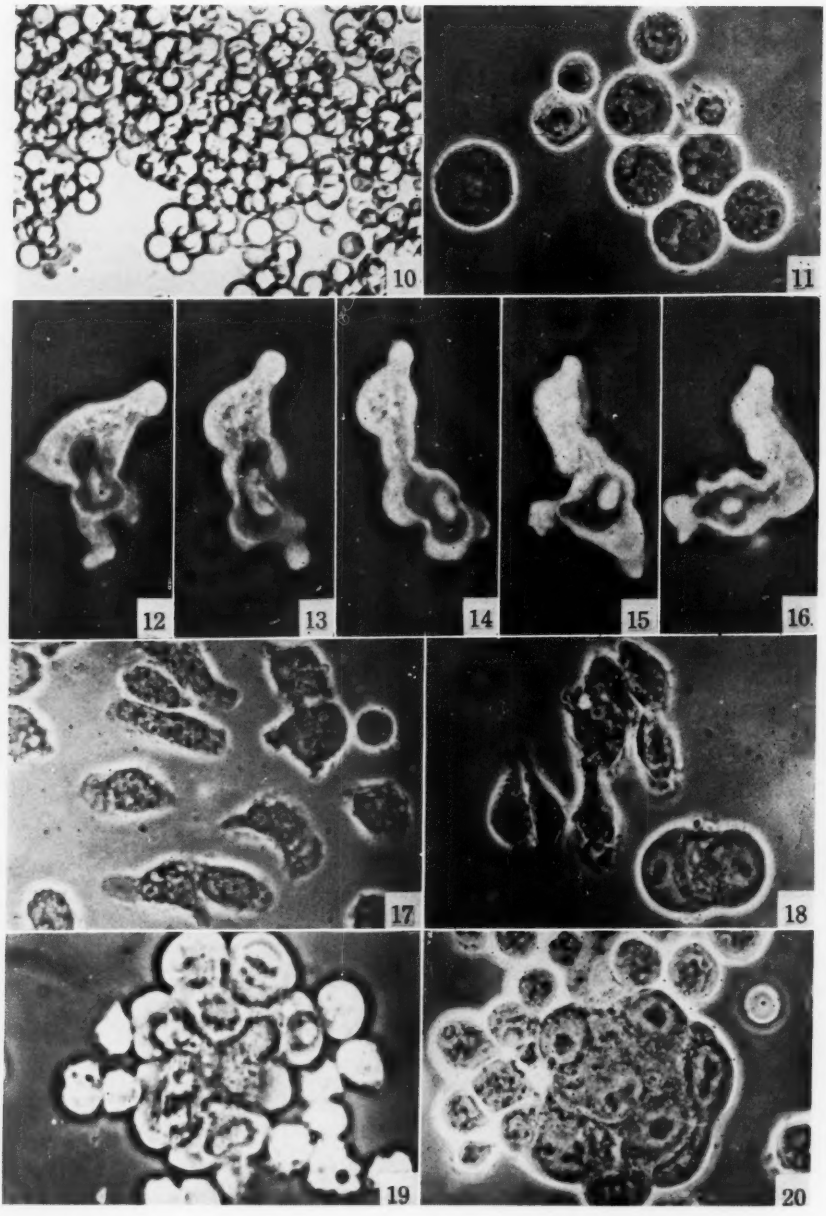
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[GANN, 50, 141~146 ; June, 1959]

SOME CYTOCHEMICAL STUDIES ON CHANGES IN MOUSE LIVER FOLLOWING THE IMPLANTATION OF CARCINOMA 51

(Plate VI)

TAKAO MATSUMOTO and SAMUEL H. HORI
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One of the previous papers dealt with the changes occurring in livers of rats bearing the ascites sarcoma and presented information on the marked depletion of glycogen, the increase of RNA- and DNA-contents, the frequent occurrence of mitosis and the sinusoidal dilatation (Hori *et al.* 1958). The present investigation was undertaken to examine cytochemical changes of livers following the subcutaneous transplantation of a tumor of a different type. In the former study the ascites sarcoma growing in the peritoneal cavity of the host was used, while in this study use was made of carcinoma 51 which develops in the subcutaneous tissue.

Material and Method: Carcinoma 51 was transplanted subcutaneously in *dd*-strain mice weighing 17 to 20 grams. The survival time of mice bearing the tumor was about 11 days. Tumor mice were sacrificed 3, 7 and 10 days after transplantation; they were conveniently refered to in description as follows:

Group A: three mice killed 3 days after transplantation
Gpoup B: three mice killed 7 days after transplantation
Group C: three mice killed 10 days after transplantation

The livers from normal and tumor-bearing mice were fixed with formal-calcium, subzero Gendre's fluid and acetic alcohol, and subjected to a series of staining methods, as described in the previous reports (Hori 1958, Hori *et al.* 1958).

OBSERVATIONS

Glycogen (PAS positive material digestable with saliva): In well-nourished normal mice, the liver glycogen was evenly distributed in the hepatic lobules. The cytoplasm of liver cells shows generally a rich content of the PAS positive substance, the amount of which was more affected by alimentary conditions than in rats (Fig. 2). The saliva extraction test of slides showed that the PAS positive substance was glycogen in nature.

The mice bearing the tumor showed decrease of glycogen content in livers without exception. In group A mice, a slight depletion of glycogen content was observed in the region around the central hepatic veins (Fig. 3), and the depletion increased

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with the passage of days. Hepatic cells in the periportal area showed a striking reduction of glycogen in mice of group B (Fig. 4).

Fnrther, almost complete or complete depletion of glycogen occurred in livers of mice carrying the tumor for 10 days or more (Fig. 5).

RNA (basophilla digestable with ribonuclease): By staining the slides with a 0.1% toluidine blue solution, liver cells of normal mice showed blue coloration in both cytoplasm and nucleoli, while their nuclear chromatin stained heterochromatically blue-violet (Fig. 13). Based on the digestion of the above slide with a 0.02% ribonuclease solution, it was found that the blue coloration after toluidine blue staining was a result of the existence of RNA in cell components. The cellular basophilia which increased in liver cells of tumor mice showed a fair degree of increase as compared with the control livers, though not so pronounced as in rat livers. In addition, a considerably strong tissue reaction seemed to occur in livers of tumor-bearing mice (Fig. 14), since many leucocytes, mostly polymorphic ones, appeared in the hepatic sinusoid and around the connective tissue. As a result, the structure of the liver was partially distorted.

Nucleoli positive to PAS reaction were found by Leuchtenberger *et al.* (1958) to occur in liver cells of mice which received intraperitoneal injection of the DNA which was extracted from the C_3H carcinoma. No such elements were detected in the present case.

DNA (microspectrophotometric determination): By means of microspectrophotometry the DNA-contents in liver nuclei of tumor-bearing mice was measured in formal-calcium fixed and Feulgen stained slides.

The results are summarized in Figure 1, with the histograms showing frequency distributions of relative amounts of DNA-contents in livers of normal mice and those of groups A, B and C. The livers of groups B and C mice showed a slight increase of DNA-contents with wider scattering of values than those of normal mice. This increment of DNA-content occurred in concomitance with an increase in volume of cell nuclei, in a manner exactly similar to

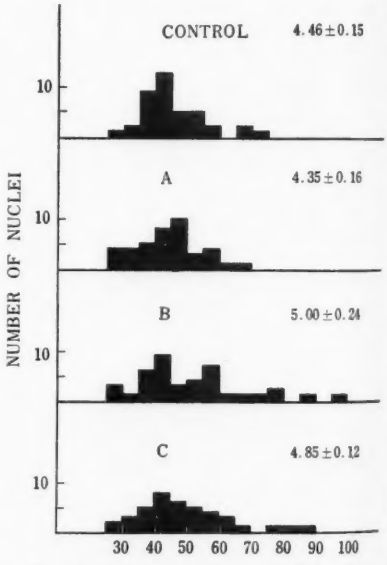

Fig. 1 Relative Amount of DNA Per Nucleus

Fig. 1. Four histograms indicate relative amount of DNA in the liver cells of normal and tumor-bearing mice sacrificed on 3, 7 and 10 days after tumor transplantation and were referred to as A, B and C in the figure, respectively.

that encountered in rats.

Mitotic frequency in the livers of rats bearing MTK-sarcoma III was relatively high (Hori *et al.* 1958), while mitosis was almost completely absent in the livers of mice bearing carcinoma 51. The results seem to suggest that the DNA-content of liver nuclei might increase independently of the mitotic activity of cells.

Further, the evidence seems to indicate that the tumor transplanted into the subcutaneous tissue of mice exerts some influence upon the host liver to raise its DNA-content.

Mitochondria: In order to detect the mitochondria, the livers from both normal and tumor-bearing animals were chromated with 3% potassium bichromate for 6 to 8 days after fixation with formal-calcium, and then stained with Regaud's iron-hematoxylin. In the livers of healthy control animals mitochondria were almost evenly distributed in the cytoplasm of liver parenchymal cells. Cells in the periportal area of hepatic lobules showed a rich content of mitochondria of voluminous rod-shape, while cells lying in the centrolobular region had filamentous mitochondria (Fig. 10). Livers from mice of groups B and C showed mitochondria, more abundantly in cells of the periportal area than in those of the centrolobular area. They were round and small granules in outline, showing less stainability than that of control cells (Figs. 11 and 12).

Acid hematein test: The cells of normal livers generally contained many cytoplasmic granules, probably mitochondria; they stained with acid hematein blue or blue black against a yellowish background (Fig. 6).

This staining situation suggests the existence of phospholipids in the granules. Cells of the periportal gegion of hepatic lobules contained mitochondria which showed slightly stronger affinity to acid hematein than did those of the centrolobular region. Such difference in stainability of mitochondria was probably due to differences in their morphology.

In liver cells of group A mice, decrease in stainability of mitochondria occurred in the centrolobular area, and then extended with the passage of days toward the peripheral portion of lobules (Fig. 7). With the decrease in stainability the granules became slender in shape (Fig. 8). In the livers of group C mice, cells of the centrolobular portion contained only a small amount of granules staining faint blue in the periphery of the cytoplasm (Fig. 9).

Discussion and Conclusion

Baldwin and Haries (1958) and Shetler *et al.* (1952) reported that polysaccharide-containing proteins of serum and serum polysaccharides associated with the albumin fraction were abundantly found in tumor-bearing animals and in cancer patients. An increase of glycolysis has also been reported in livers of leukemic mice by Hall

(1944) and Burk *et al.* (1942). Comparing the glucose and lactic acid contents of the blood from the axillary veins of a chicken bearing Rous sarcoma, Cori and Cori (1925) have found less glucose and more lactic acid in the vein draining the tumor. Warburg *et al.* (1926) also observed a great difference in lactic acid and glucose contents between arterial and venous blood of rats bearing the Jensen sarcoma; these findings indicate an active utilization of glucose by the tumor, with production of lactic acid. The present study has shown that all the mice carrying the tumor for 10 days or more showed a complete depletion of glycogen in their livers. On the basis of the above findings it is possible to speculate that liver glycogen is taken into the blood stream in order to meet the enormous requirement of energy by tumor cells. Goranson *et al.* (1954) have obtained results contradictatory the above view in that the levels of liver glycogen were essentially the same between fasted control and tumor-bearing rats and that there was no real difference in the phospholylase activity of liver between the control and tumor rats. In the present study, the authors examined the stomachs of tumor mice when sacrificed and found that they were filled with food. It is therefore not likely that the depletion of liver glycogen in tumor mice was primarily caused by anorexia which was often said to be due to the presence of the tumor.

Chemical analysis using labelled precursors have revealed evidence which indicates an increased rate of nucleic acid synthesis in the tissue of tumor-bearing mice (Kelly and Jones 1950, Payne *et al.* 1952 a and b, Anderson *et al.* 1955). Khouvine and Mortreni (1954) have pointed out the possible existence of a substance which was derived from the tumor and exerted influence upon the nucleic acid synthesis of the liver. A significant increase of DNA-content was also observed by the present study in livers of mice bearing tumor transplants, exactly the same as is seen also in those of rats bearing tumors. There was, however, little mitosis in livers of tumor-bearing mice, in striking contrast to the frequent occurrence of mitosis in livers of tumor-bearing rats. Then, it seems probable that the increase of DNA-content might have taken place, independently of the mitotic events, due probably to the abnormal metabolic activity of liver cells induced by the presence of tumors.

It has been shown that the morphology of the mitochondria undergoes alterations under various physiological, pathological and experimental conditions (Lewis and Lewis 1945, Smith 1931, Weathereford 1933, Gey *et al.* 1955, Hori 1958). Hori (1958) has found in rats that mitochondria of hepatic cells of the centrolobular area transform in shape from filament into fine-spun threads and give up affinity to acid hematein, following cortisone administration. Similar observations were made during the present study in livers of tumor-bearing mice. Then it is likely that filamentous mitochondria of liver cells of the centrolobular area tend to loose affinity to acid hematein under certain conditions.

Summary

Carcinoma 51 was transplanted subcutaneously into mice of *dd*-strain. The changes in content of glycogen, RNA, DNA and phospholipids in the livers, as well as alteration in mitochondrial morphology, were studied by a series of cytochemical staining and cytophotometric methods.

The results obtained indicate that: 1) glycogen is gradually depleted in livers of tumor-bearing mice and becomes completely absent when mice were moribund; 2) the DNA-content in liver cells of tumor-mice shows an increase 7 and 10 days after tumor-transplantation, in concomitance with the increase of nuclear volume of liver cells; 3) RNA was found to be rather rich in livers of some tumor-mice; 4) the phospholipid-content of liver cells as revealed by the acid hematein test shows a decrease specially in the centrolobular area of the liver, and 5) filamentous mitochondria in cells of the centrolobular area tend to break down into granules, and loose phospholipids.

Acknowledgement

The authors wish to express their deep appreciation to Professor Sajiro Makino for his keen interest in this study and for going through this paper before its publication. Further thanks must be extended to Professor Y. Ojima, Kwansei Gakuin University, who kindly permitted us to make the DNA measurements in his laboratory.

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Explanation of Plate VI

Figs. 2-5. Fixative: subzero Gendre's fluid. Stain: Periodic acid Schiff. Mgnif. 200×.

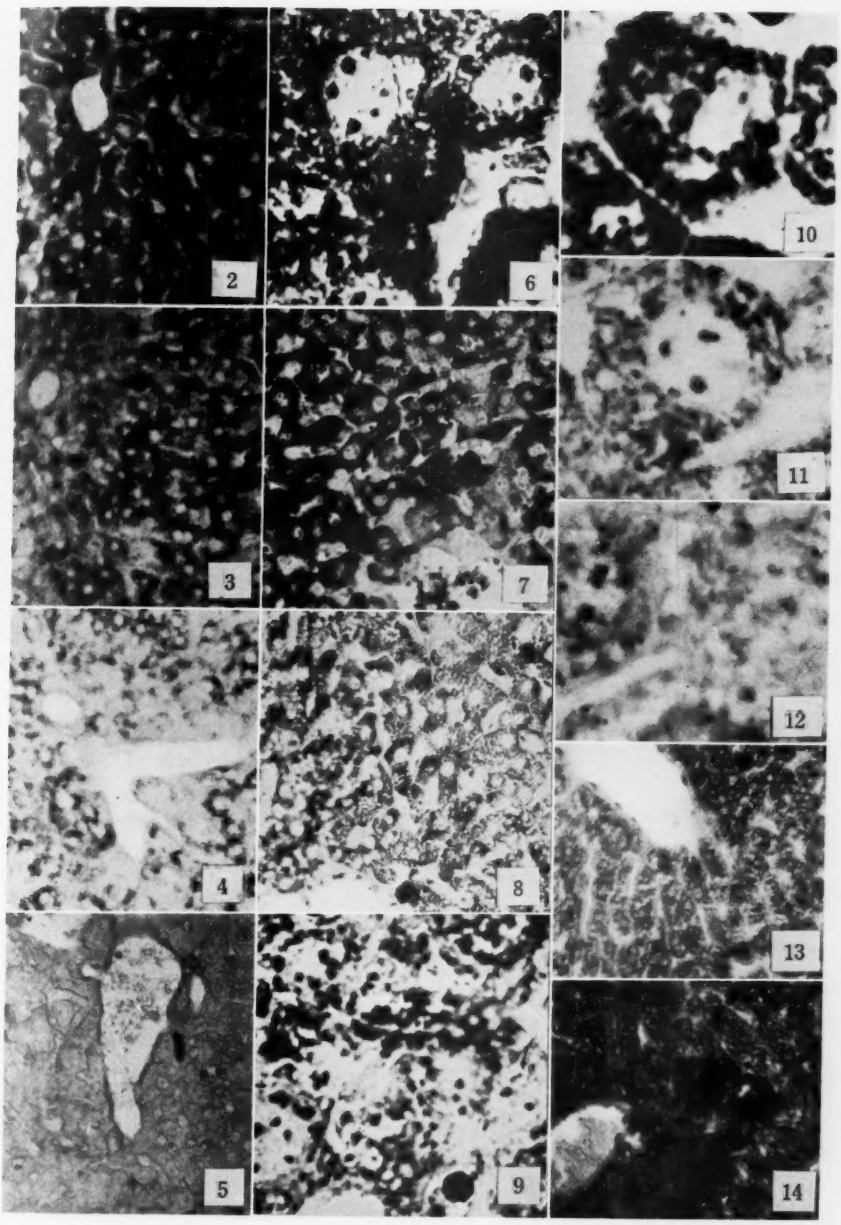
Fig. 2. Control mouse liver.

Figs. 3-5. Livers of tumor-bearing mice sacrificed on 3, 7 and 10 days after transplantation, respectively. Note the gradual diminution of PAS positive substance in the liver parenchymal cells.

Figs. 6-9. Fixative: formal-calcium. Stain: acid hematein. Fig. 6. Control mouse liver, showing strongly stained granules, probably mitochondria. 400×. Figs. 7-9. Livers from tumor-bearing mice killed 3, 7 and 10 days after tranplantation, respectively. Magnif. 100×, except 400× in Fig. 9.

Figs. 10-12. Fixative: formal-calcium. Stain: Rgaud's iron-hematoxylin. Magnif. 1800×. Fig. 10. Normal liver cells. Figs. 11 and 12. Liver cells of tumor-bearing mice killed 7 and 10 days after tumor transplantation, respectively. Mitochondria of cells in centrolobular area show marked morphological alteration and decrease in stainability.

Figs. 13-14. Fixative: formal-calcium. Stain: toluidine blue. Magnif. 100×. Fig. 13. Normal mouse liver. Fig. 14. Tumor liver from the mice killed 10 days after tumor transplantation. Note the increase in cellular basophilia and many leucocytes around the interlobular vein.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GANN, **50**, 147~154; June, 1959]

CROSS-RESISTANCE BETWEEN MITOMYCIN C AND ALKYLATING AGENTS IN EXPERIMENTAL CANCER CHEMOTHERAPY

SHOICHI OBOSHI

(Department of Pathology, Hirosaki University School of Medicine)

The development of resistance to chemotherapeutic agents is a familiar phenomenon in most of infective diseases. The similar phenomenon is also known in the field of cancer chemotherapy. The development of several lines of neoplastic cells showing a resistance to and, on rare occasions, a dependence on certain antitumor substances has been now established, and the development of cross-resistance is also observed through the use of antimetabolites or alkylating agents.[1]

In this paper, the development of a variant of Hirosaki sarcoma resistant to mitomycin C, effects of several antitumor agents on this subline and an effect of mitomycin C upon the resistant subline of Hirosaki sarcoma developed through the use of nitrogen mustard N-oxide are reported.

MATERIALS

All animals used in this experiment were male or female hybrid rats weighing about 100 g.

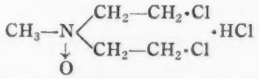
Diploid Hirosaki sarcoma, that is, an ascitic lymphosarcoma of rat, was used. This strain established in our laboratory in 1951 and successively transferred by intraperitoneal transplantation, is thought to be similar to Yoshida sarcoma in its biological and cytological properties.[2]

Antitumor agents used in the present study are as follows:

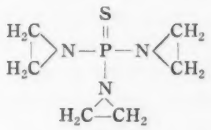
Mitomycin C: This is a new antitumor antibiotic found by Hata[3] and Wakaki.[4] Effects of this antibiotic upon various animal tumors were reported previously by Usubuchi.[5,6]

Carzinophilin and actinomycin J: These are also antitumor antibiotics, of which the former was reported by Hata[7] and the latter by Nishibori.[8]

Nitrogen mustard N-oxide (MBAO) and triethylene thiophosphoramide (TSPA): Both of these compounds are alkylating agents in popular use, having the formula as follows:



MBAO



TSPA

Methods and Results

1. Establishment of a subline of Hirosaki sarcoma resistant to mitomycin C.

It was previously reported that tumor cells of the animals consecutively treated with the doses more than 50 mcg/kg/day of mitomycin C disappeared from the ascites, and the hosts showed a marked prolongation of survival days or complete cure. In the present study a small amount of mitomycin C, 5–10 mcg/kg/day, was daily injected intraperitoneally starting from 48 hours after transplantation. Tumor cells in the treated animal proliferated in the same degree as in the untreated animals, and the animal died of tumor invasions without showing any prolongation of survival period. Enormous tumor cells with polymorphous nuclei and lace-like vacuoles at the margin of cytoplasms were seen in a small number. Administering daily small doses of mitomycin C, successive transplantations were performed. After the fifth generation, that is 36 days after the first injection of mitomycin C, the transferred ascitic tumor cells proliferated in the ascites without showing any degenerative change and the host animal died of tumor invasions without showing any prolongation of survival period, even though they were treated consecutively with the effective doses, 100 mcg/kg/day, of mitomycin C. (Fig. 1)

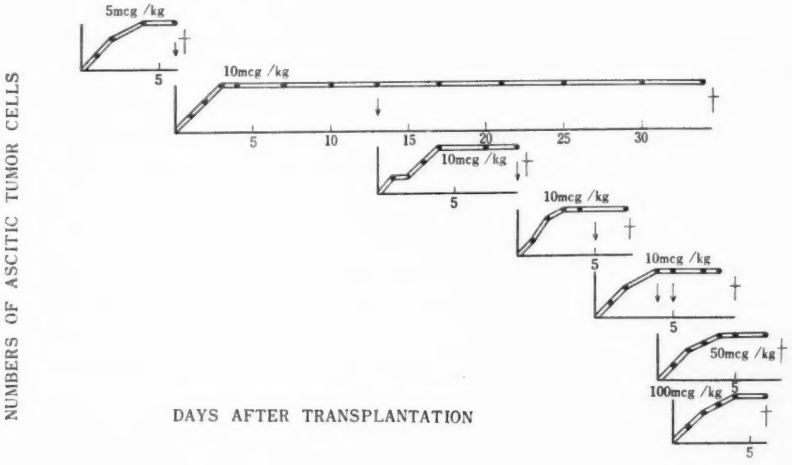


Fig. 1. Successive transfers for development of resistance to mitomycin C in Hirosaki sarcoma.

| = 400,000 sarcoma cells/cmm ascitic fluid. In this and all other figures in this paper, the number of ascitic tumor cells is expressed in the same way.

↓ = day of transplantation to next animal.

+ = day of death.

A subline completely resistant to 200 mcg/kg/day of mitomycin C was established through successive transfers of the tumor cells and, at the same time, by treating the animals with the gradually increasing doses of the agent. The resistance of the tumor cells of this subline to mitomycin C remained unvaried for more than two months after the use of the agent was stopped. Transplantability and cytological properties of this variant were identical with those of the original line.

2. Effects of other antitumor substances upon the subline resistant to mitomycin C.

MBAO, TSPA, carzinophilin and actinomycin J were used. Generations from 10 to 14 of the subline were employed to test the effects of these antitumor substances. At the same time, their effects upon the original line of Hirosaki sarcoma were also tested as control.

a) Effect of MBAO

By consecutive treatment with the doses more than 1 mg/kg/day of MBAO, tumor cells of the original line disappeared from the ascites and the host animals showed a marked prolongation of survival days or complete cure without exception. But, in cases of the resistant subline, the ascitic tumor cells increased as well as those of untreated animals, though the toxic doses, 20mg/kg/day, of MBAO were consecutively administered starting just after transplantation. (Fig. 2)

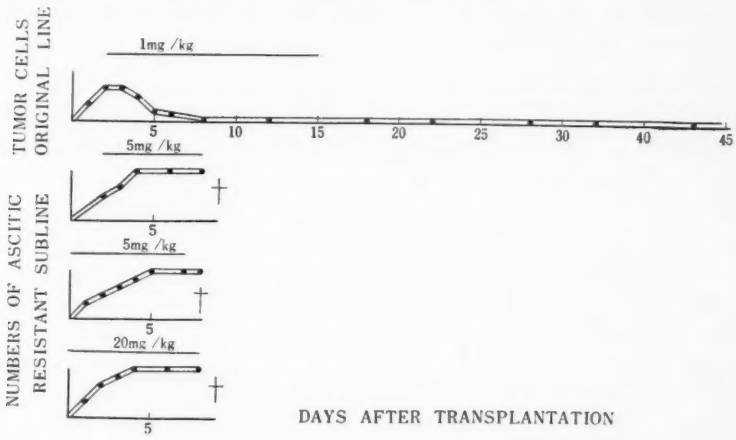


Fig. 2. Comparison of effects of MBAO upon the original line and mitomycin-resistant subline of Hirosaki sarcoma.

b) Effect of TSPA

The minimal effective doses of TSPA upon the original line were 0.5–1.0mg/kg/day. On the contrary, the daily administration of the toxic doses, 10 mg/kg/day, of this agent had neither cellular nor life-prolonging effect upon the subline resistant to mitomycin C. (Fig. 3)

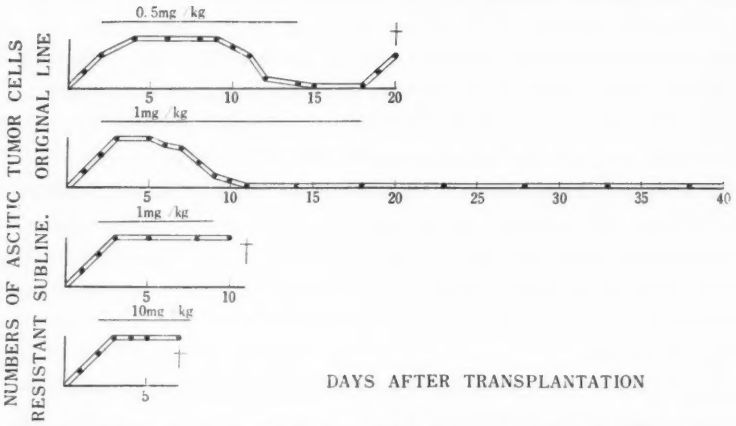


Fig. 3. Comparison of effects of TSPA upon the original line and mitomycin-resistant subline of Hirosaki sarcoma.

c) Effect of carzinophilin

A proliferation of ascitic tumor cells of both the original and resistant line was slightly inhibited by treatment with 2,500 u/kg/day of carzinophilin and markedly inhibited by treatment with 5,000 u/kg/day of this antibiotic. (Fig. 4)

d) Effect of actinomycin J

An increse of tumor cells in the resistant subline, which were treated with consecutive injection of 20–50 mcg/kg/day of actinomycin J beginning just after trans-

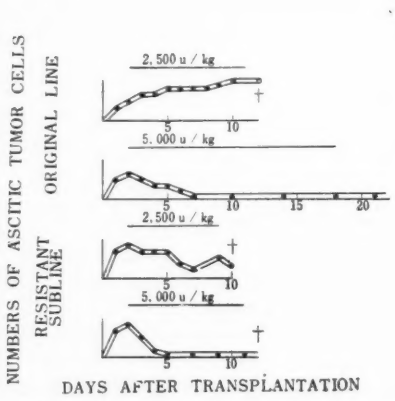


Fig. 4. Comparison of effects of carzinophilin upon the original line and mitomycin-resistant subline of Hirosaki sarcoma.

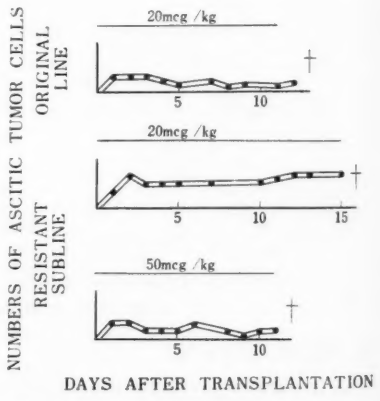


Fig. 5. Comparison of effects of actinomycin J upon the original line and mitomycin-resistant subline of Hirosaki sarcoma.

plantation, was inhibited in the same degree as in the similarly treated original line. (Fig. 5)

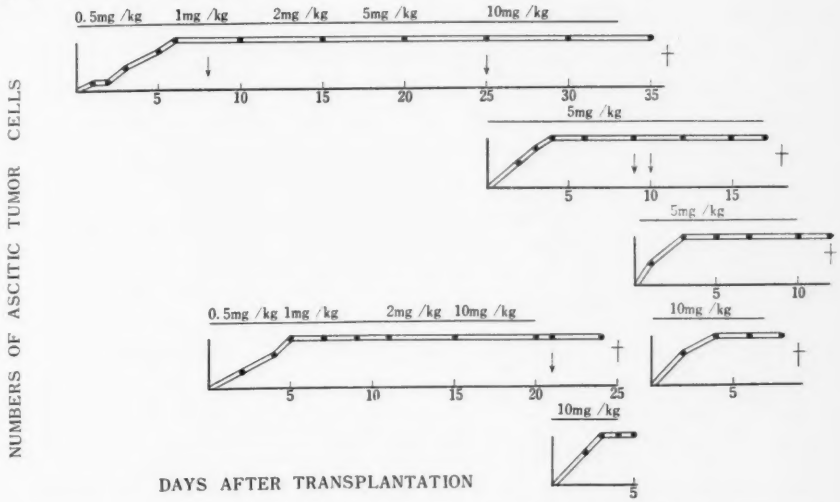


Fig. 6. Successive transfers for development of resistance to MBAO in Hirosaki sarcoma.

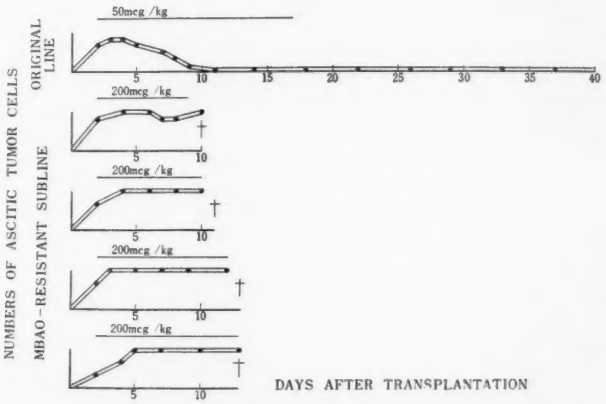


Fig. 7. Comparison of effects of mitomycin C upon the original line and MBAO-resistant subline of Hirosaki sarcoma.

3. Effect of mitomycin C on the MBAO-resistant subline of Hirosaki Sarcoma. Previously the author[9] reported that a subline of Hirosaki sarcoma resistant to MBAO was easily established by consecutive injections of the effective doses, 1-5mg/kg/day, of MBAO. In the present study the MBAO-resistant subline was established by the same way as the subline resistant to mitomycin C. Tumor cells of this sub-

line were never affected by consecutive treatment with the toxic doses, 10mg/kg/day, of MBAO starting just after transplantation. (Fig. 6)

By consecutive treatment with 50mcg/kg/day of mitomycin C, tumor cells of the original line disappeared from the ascites and the host animals almost escaped death. But, the MBAO-resistant subline never improved by treatment with 200 mcg/kg/day. (Fig. 7)

Discussion

The conversion of a tumor normally sensitive to a drug into one which is refractory, is of particular interest in relation to investigation in the field of microorganisms. It is said that the studies on cancer chemotherapy are not only of practical use, but also may answer the purpose to master a nature of neoplasm.[10] Observations of transplantable fluid tumors, such as leukemias and ascitic tumors, set the author's mind to the parasitic concept of neoplasm. The development of resistance to a drug in cancer chemotherapy may be also considered as one of favorable evidences for a parasitic concept of cancer.

In 1950 Burchenal[11] first described the phenomenon of resistance to A-methopterin, which developed in leukemic cells of a lymphocytic neoplasm AK 4 in mice. In the same year, Law[12] reproted that the sublines resistant to and dependent on various 4-amino substituted folic acid analogs were established using the lymphocytic neoplasm L 1210 in mice. Since then numerous resistant and dependent variants developed particularly in the lymphomas have been reported.

Folic acid antagonists, purine antagonists and nitrogen mustard derivates have been popularly used in these studies. Previously the author[13,14] also described the establishment of a subline of Hirosaki sarcoma resistant to MBAO. On the other hand, in the studies using the antibiotics, only one paper, which showed that variants of plasma cell leukemia 70429 resistant to and dependent on azaserine were established, was reported by Potter[15] in 1956.

Various methods have been used to test the development of resistance. Burchenal[11] used a method of average survival days to discriminate the development of resistance of leukemia AK 4 to A-methopteron. Law[16] employed the mean weight of the subcutaneously transplanted lymphoma tissue as a criterion of the development of resistance to folic acid antagonists. Hirono[17] principally used the number of tumor cells and mitotic figures and their morphological findings in his study on the development of resistance of Yoshida sarcoma to MBAO. In the author's present study, both cellular changes and life-prolongation effects were favorably used as criteria. It is well known that the survival periods of animals in various ascitic tumors are fixed. The cellular effect of mitomycin C on tumor cells of Hirosaki sarcoma is characteristic.[18]

Changes of the ascitic tumor cells observed after a single intraperitoneal injection of 200mcg of mitomycin C are briefly described as follows; Mitotic figures began to decrease in number 3 hours after injection of this agent and were scarcely seen in the ascites 12 hours after injection. 48–72 hours after injection mitotic figures increased again in the ascites, but morphologically they were abnormal ones. Most of the chromosoms of these cells were characterized by being multiplied, cut off, scattered or bridge-shaped. The resting cells remained morphologically unaffected until 12 hours after injection, but thereafter they showed degenerative features characterized by enormous cells with polymorphous nuclei and lace-like vacuoles at the margin of the cytoplasms. The ascitic tumor cells markedly decreased in this stage. By treating with mitomycin C, however, resistant tumor cells increased in number without showing any of the above changes.

It is popularly known in the field of chemotherapy of microorganisms that a resistant variant developed through the use of one of sulfonamides shows a cross-resistance to all sulfonamide derivates, and a similar phenomenon is also observed by the use of tetracyclines. In studies on cancer chemotherapy, cross-resistance to all 4-amino substituted folic acid antagonists has been known for the L 1210 variants developed through the use of these compounds. Similarly cross-resistance was shown to all purine antagonists studied for the L 1210 variants developed through the use of 8-azaguanine, 6-mercaptopurine and 6-thioguanine. Hirono[19] described that a subline of Yoshida sarcoma resistant to MBAO showed a cross-resistance to three other alkylating agents, methylbis, tris and TEM, but not to unrelated substances such as colchicine and 8-azaguanine or to X-ray.

In the present study, cross-resistance to MBAO and TSPA was shown for the resistant variant of Hirosaki sarcoma developed through the use of mitomycin C. And moreover, a variant developed through the use of MBAO also showed a cross-resistance to mitomycin C. But, the sensitivity of this variant line to other unrelated antibiotics such as actinomycin J and carzinoplilin remained similar to that of the original line.

From these results, the mechanism of antineoplastic activity of mitomycin C seems to be similar to that of alkylating agents such as MBAO and TSPA.

Summary

By consecutive injections of mitomycin C and MBAO, sublines of Hirosaki sarcoma resistant to each agent respectively were obtained. A variant developed through the use of mitomycin C showed a cross-resistance to MBAO and TSPA, but not to other unrelated antibiotics. Moreover, a variant developed through the use of MBAO showed a cross-resistance to mitomycin C.

From these results the author concluded that the mechanism of antitumor activity of mitomycin C might be similar to that of alkylating agents.

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EFFECT OF p-DIMETHYLAMINOAZOBENZENE ON THE EXCRETION OF B VITAMINS IN THE URINE OF RAT

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It has been well known that the riboflavin content of the liver of rats was reduced on the inclusion of azo dyes in the diet and the more potent carcinogenic dyes effected the greater reduction of riboflavin in the liver (1, 2).

Recently, Doi (3) has inquired into the riboflavin content of liver of rats fed p-dimethylaminoazobenzene (DAB) throughout the period of tumor induction and found that the greatest reduction of riboflavin in the liver was observed at the end of first month of DAB feeding and then the flavin content gradually increased up to the period of tumor formation.

This time curve of riboflavin content in the liver of rats fed DAB shows the reverse situation to that of polar dye formation (4). These facts might imply the protein which binds with DAB to be a flavoprotein of some kind.

At this stage, however, it is also worthwhile to know the effect of DAB on the contents of the other coenzymes in the body. This communication offers data on the effect of azo dyes on the urinary excretion of some B vitamins.

Materials and Methods

Animals: Albino rats weighing 150-200g were fed on DAB or 4-aminoazobenzene (AB) coated rice as usual (5). For control, rats were fed on rice only. The twenty-four hour urine of rats was collected every week end for 4 weeks, and the amounts of B vitamins in the urine were determined.

Determination of B Vitamins: Thiamine was determined by the method of Fujiwara and Matui (6). Total riboflavin was estimated by the fluorophotometric method of Yagi (7), niacin assayed by the method of Swaminathan (8), and pyridoxic acid determined by the fluorophotometric method of Fujita et al. (9).

Paper Chromatography: For the confirmation of lumiflavin or riboflavin, two dimensional chromatography was applied. Butanolacetic acid- water (250-60-250 v/v) and 5% Na_2HPO_4 were the solvents used. Papers used were Toyo-Roshi No. 50. Spots were detected by ultraviolet irradiation.

Results

The Excretion of Thiamine in the Urine of Rats fed DAB or AB. A slight

increase of total thiamine in the liver of rats fed DAB has been reported by Masayama and Yokoyama (10). They also have stated that free thiamine was increased and cocarboxylase was decreased as compared with normal.

Urinary excretion of thiamine had no remarkable difference between normal and azo dye groups of rats as shown in Table 1 and Chart 1. These results indicate that

Tabel 1. Excretion of Thiamine in the Urine of Rats fed Azo Dyes.

feeding Period \ fed Rats		DAB	AB	Control
End of 1st week	Volume of Urine (ml.)	2.7	2.1	1.3
	Amount of free Thiamine (γ/day)	0.42	0.27	0.27
End of 2nd week	Volume of Urine (ml.)	3.8	3.3	0.8
	Amount of free Thiamine (γ/day)	0.5	0.36	0.45
End of 3rd week	Volume of Urine (ml.)	1.8	1.5	1.5
	Amount of free Thiamine (γ/day)	0.21	0.12	0.03
End of 4th week	Volume of Urine (ml.)	1.8	1.6	1.1
	Amount of free Thiamine (γ/day)	0.26	0.25	0.18

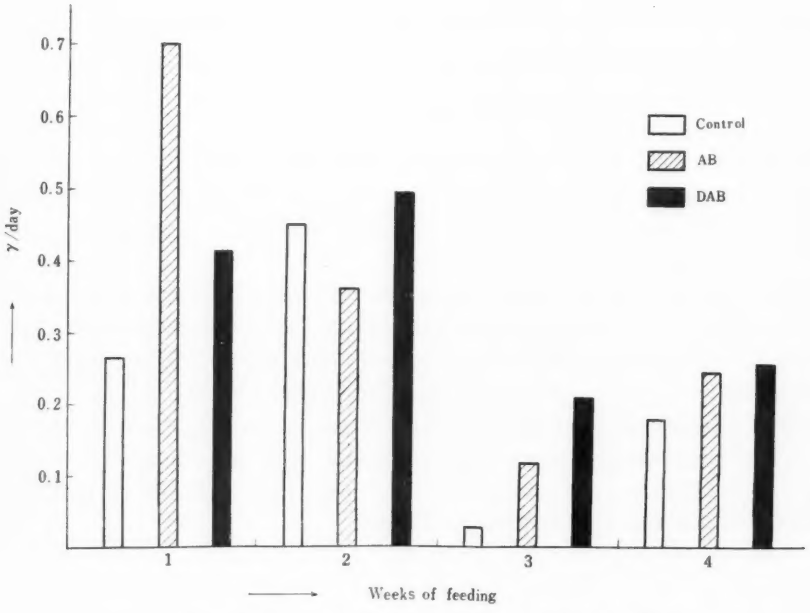

Chart 1. Excretion of Thiamine in the Urine of Rats fed Azo Dyes

feeding of rats on azo dyes has no influence on the thiamine content in the animal body.

The Excretion of Niacin in the Urine of Rats fed Azo Dyes. Kensler et al. (11) have reported that the content of diphosphopyridine nucleotide (DPN) in the liver of rats fed DAB decreased considerably. Although the increased excretion of niacin in the urine of rats fed azo dyes was observed (Table 2 and Chart 2), the extent of increment was far smaller than that of ribofiavin as shown in Table 4. Furthermore,

Table 2. Excretion of Nicotinic Acid in the Urine of Rats fed Azo Dyes

feeding Period \ fed Rats		DAB	AB	Control
End of 1st week	Volume of Urine (ml.)	2.2	2.8	1.1
	Amount of Nicotinic Acid (γ/day)	28.0	24.8	11.5
End of 2nd week	Volume of Urine (ml.)	3.3	1.8	1.5
	Amount of Nicotinic Acid (γ/day)	42.0	23.5	13.0
End of 3rd week	Volume of Urine (ml.)	1.6	3.2	0.5
	Amount of Nicotinic Acid (γ/day)	29.5	21.0	18.5
End of 4th week	Volume of Urine (ml.)	2.6	2.0	1.6
	Amount of Nicotinic Acid (γ/day)	40.5	43.0	30.5

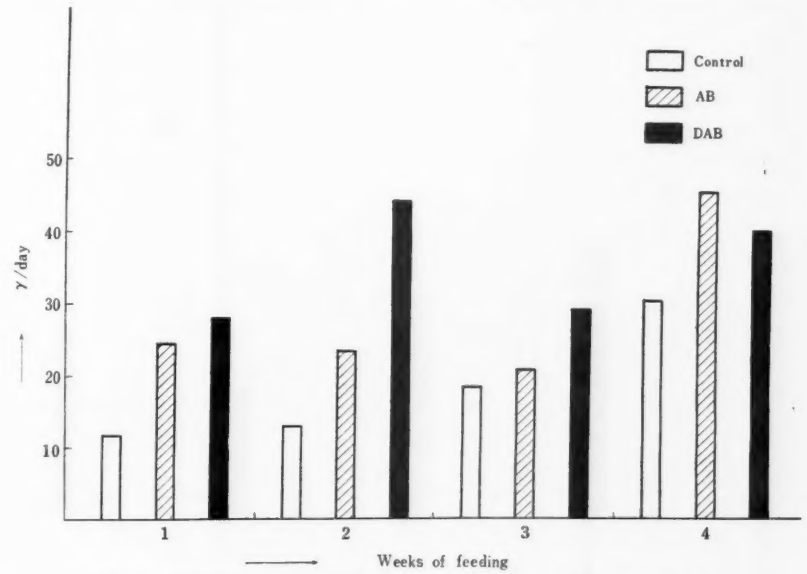

Chart 2. Excretion of Nicotinic Acid in the Urine of Rats fed Azo Dyes

the daily volume of urine of rats fed azo dyes was always higher than that of normal rats fed rice only, so the niacin content per ml of urine was almost the same in both.

The Excretion of Pyridoxic acid in the Urine of Rats fed Azo Dyes. Price et al. have stated that the ingestion of DAB reduced the vitamin B_6 content of rat liver (12). The amount of urinary pyridoxic acid, however, was not increased, but rather reduced by feeding on azo dyes (Table 3 and Chart 3). The reason for this discrepancy is not apparent at the present time. Among the pyridoxine analogues which appeared in the urine, pyridoxic acid was predominant.

Table 3. Excretion of Pyridoxic Acid in the Urine of Rats fed Azo Dyes

Feeding Period \ Fed Rats		DAB	AB	Control
End of 1st week	Volume of Urine (ml.)	2.5	3.6	1.0
	Amount of Pyridoxic Acid (γ/day)	3.8	3.0	3.7
End of 2nd week	Volume of Urine (ml.)	1.0	1.0	0.6
	Amount of Pyridoxic Acid (γ/day)	3.1	1.6	5.0
End of 3rd week	Volume of Urine (ml.)	2.8	0.8	0.5
	Amount of Pyridoxic Acid (γ/day)	1.8	0.9	3.7
End of 4th week	Volume of Urine (ml.)	3.2	0.7	0.8
	Amount of Pyridoxic Acid (γ/day)	3.1	2.0	4.5

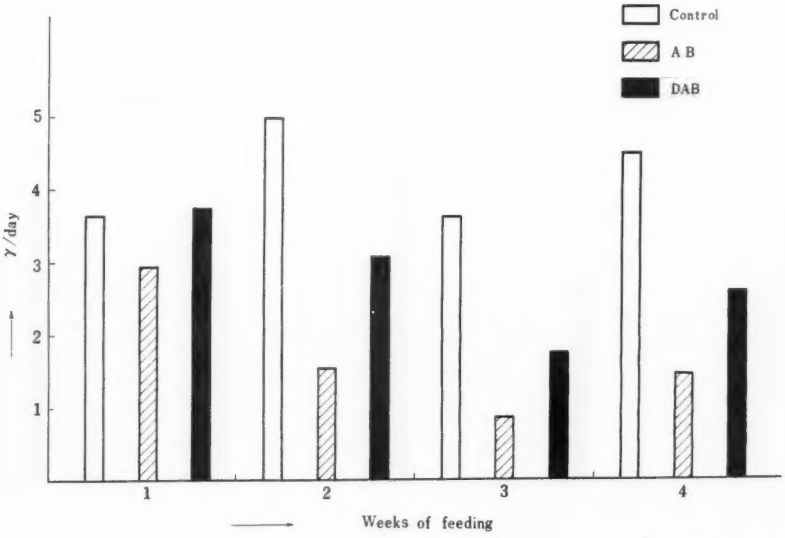

Chart 3. Excretion of Pyridoxic Acid in the Urine of Rats fed Azo Dyes

The Excretion of Riboflavin in the Urine of Animals fed Azo Dyes. In contrast to the other B vitamins above examined, the urinary ribofiavin was markedly affected by the ingestion of DAB (Table 4 and Chart 4). At the end of third week, for example, the amount of urinary ribofiavin excreted by rats fed DAB was thirty times as high as that excreted by rats fed rice only.

Such remarkable effect of DAB on the fiavin ejection, however, was not observed

Table 4. Excretion of Riboflavin in the Urine of Rats fed Azo Dyes.

feeding Period \ fed Rats		DAB	AB	Control
End of 1st week	Volume of Urine (ml.)	1.5	1.2	0.4
	Amount of total Riboflavin (γ/day)	6.8	1.3	0.2
End of 2nd week	Volume of Urine (ml.)	1.6	1.2	1.3
	Amount of total Riboflavin (γ/day)	1.9	1.7	0.3
End of 3rd week	Volume of Urine (ml.)	1.0	1.5	0.8
	Amount of total Riboflavin (γ/day)	3.6	2.0	0.1
End of 4th week	Volume of Urine (ml.)	0.8	0.4	0.7
	Amount of total Riboflavin (γ/day)	1.4	0.2	0.2

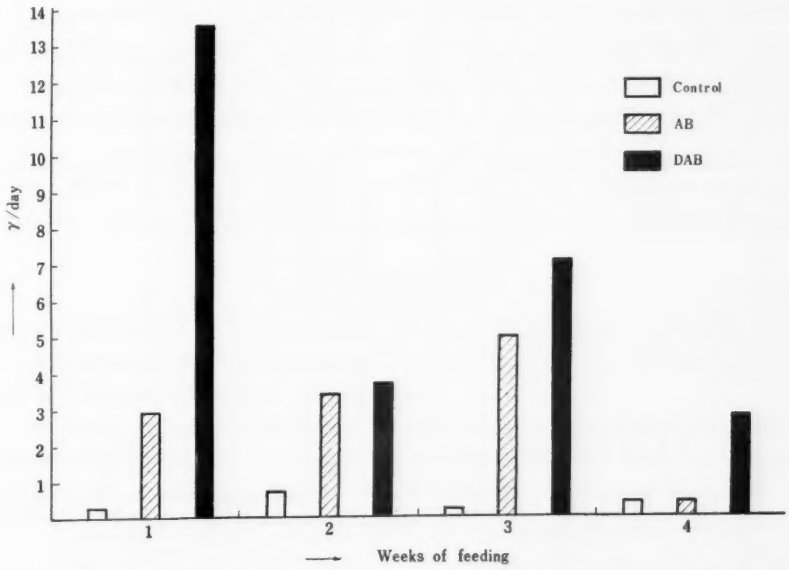

Chart 4. Excretion of Riboflavin in the Urine of Rats fed Azo Dyes

in the case of guinea pig known to resist the azo dye tumor induction (Table 5). These facts may indicate the relation between the reduction of riboflavin and the tumor incidence. But Spain and Clayton (13) reported that thorotrast and colloidal iron oxide inhibited the tumor induction by DAB in the rat liver, but not prevented the reduction of liver riboflavin by DAB.

Table 5. Excretion of Riboflavin in the Urine of Guinea pig fed Azo Dye.
(At the end of one week of feeding)

DAB fed Guinea Pig		Control	
Volume of Urine (ml.)	Amount of total Riboflavin (γ/day)	Volume of Urine (ml.)	Amount of total Riboflavin (γ/day)
5.0	0.9	6.6	3.2
5.2	7.6	4.8	2.7
6.0	13.0	3.4	9.0

Recently, Salzberg (14) has reported that when the livers of rats fed labeled 3′-$C^{14}H_3$-DAB for 21 days were analysed for riboflavin, a radioactive component has always accompanied the riboflavin in various chemical procedures for the isolation of flavin compound. But this radioactive compound derived from 3′-methyl-DAB had no fluorescence and the Rf. value of the labeld compound was slightly higher than that of riboflavin on a paper chromatogram (Butanol-Acetic acid-Water).

Although riboflavin in the urine was determined by the lumiflavin method in the present experiments, further confirmation of urinary riboflavin was carried out by paper chromatography. Chloroform solution of lumiflavin extracted from the urine was applied on paper. Charts 5, 6 and 7 show the chromatograms of lumiflavin

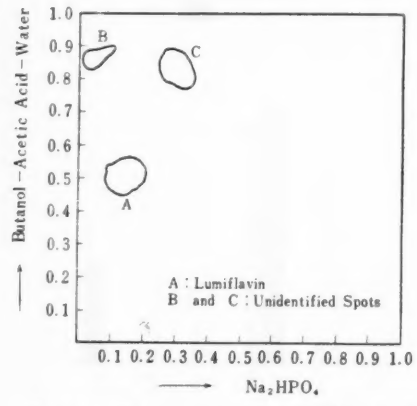

Chart 5. Chromatogram of Lumiflavin of the urine of rats fed DAB for 3 weeks

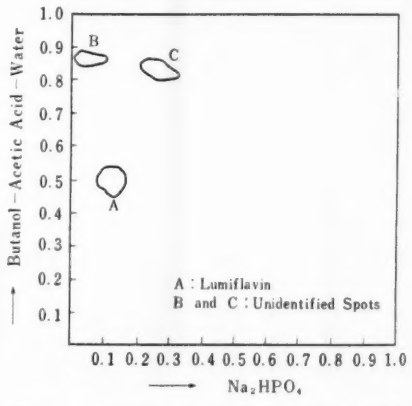

Chart 6. Chromatogram of lumiflavin of the urine of rats fed AB for 3 weeks

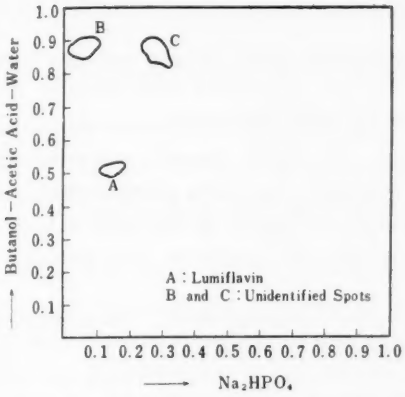

Chart 7. Chromatogram of lumiflavin of the urine of rats fed rice for 3 weeks

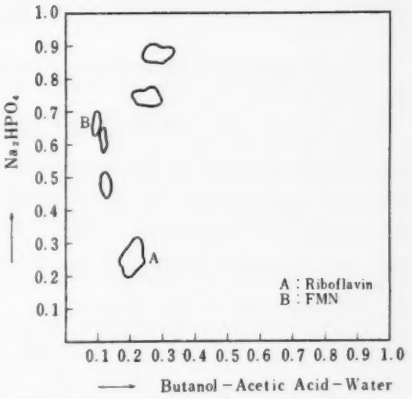

Chart 8. Chromatogram of the urine of DAB fed rat (for 2 week)

fraction in the urine of rats fed DAB, AB and rice only.

Three spots were detected by the ultraviolet irradiation. Spot A was confirmed to be lumiflavin by using authentic sample (15). Spot A on these three papers, after cutting out from the chromatograms, were dissolved in hot water and determined fluorophotometrically. The ratio of amounts of lumiflavin thus determined on these spots agreed with that determined by the usual chemical method of Yagi.

In order to know the type of flavin compounds which appeared in the urine, a flavin solution prepared without alkali photolysis from the urine of rats fed DAB was chromatographed on paper with the same solvents as used above. By using authentic samples spots A and B on chart 8 were confirmed to be riboflavin and flavin mononucleotide (FMN) respectively. Flavin adenine dinucleotide (FAD) was not detected. The ratio of riboflavin : FMN was determined to be about 4 : 1 by cutting out method.

Discussion

The profound effect of DAB on the flavin ejection appears to imply that the first event which occurs after the ingestion by the rats of DAB may be the competition betwen azo dyes and flavins to a flavoprotein.

If more daring speculation be permitted, the protein binding with the azo dyes and giving the polar dye might be a flavoprotein of some kind. As pointed out by Spain and Clayton (13), the flavin defficiency induced by DAB ingestion in the animal body may not be an essential factor for the tumor induction, but still somewhere around this point there appears to be an important key for the settlement of the problem of cancer induction.

Summary

The amounts of B vitamins in the urine of rats fed DAB, AB and rice only were determined.

The concentration of thiamine, niacin, and pyridoxic acid in the urine showed no remarkable changes by the ingestion of azo dyes. Riboflavin, however, was amazingly increased in the urine of rats fed DAB. AB had also some flavin ejection effect, but this effect was weaker than DAB. The amount of riboflavin in the urine of guinea pig, which resists tumor induction by azo dyes was not affected by DAB feeding.

The author wishes to thanks Prof. S. Mizuhara for his helpful suggestions. This investigation was aided in part by a research grant from the Ministry of Education.

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CHANGES OF SOME FLAVIN ENZYMES IN THE LIVER OF RAT DURING DAB CARCINOGENESIS. I.

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When rats were fed on p-dimethylaminoazobenzene (DAB), the reduction of liver riboflavin, the increased excretion of this vitamin in the urine, and the reverse situation between the time curve of riboflavin content and of polar dye production in the liver were observed in the previous experiments (1), (2).

These facts might imply the protein bound with DAB to be a flavoprotein of some kind, and it became of interest to inquire which flavoprotein was affected significantly during DAB-carcinogenesis.

This paper describes the effects of DAB feeding on the activities of D-amino acid oxidase, xanthine oxidase, and succinic dehydrogenase in the liver.

Materials and Methods

Albino rats weighing 150-200g were fed for about one month on DAB-coated rice as usual (3). The control rats were fed on rice coated with olive oil only.

The animals which were used for the assay were decapitated, and exsanguinated. The livers were removed and placed immediately in cracked ice. The weighed livers were homogenized with a glass homogenizer in a suitable volume of water or phosphate buffer.

For the determination of D-amino acid oxidase a manometric method described by Burton (4) was employed with a slight modification. Xanthine oxidase in the liver was assayed by the method of Litwack et al. (5). A manometric method described by Singer and Kearney (6) was used for the determination of succinic dehydrogenase activity in the liver.

Phenazine methosulfate used was kindly furnished by Dr. D.E. Green of Wisconsin (U.S.A.).

Results

1) *D-Amino Acid Oxidase*

One ml of water homogenate of liver was pipetted into each of four Warburg flasks containing the following mixtures:

Flask No.	0.1M-Pyrophosphate Buffer (pH 8.3)	10^{-4}M FAD	5%D,L-Alanine (side arm)	Water
1	1.0 ml	0.1 ml	0.1 ml	—
2	1.0 ml	—	0.2 ml	0.1 ml
3	1.0 ml	—	—	0.3 ml
4	1.0 ml	0.1 ml	—	0.2 ml

In this experiment, catalase was not added into the mixture. As shown in Table 1, 42 to 93μl of oxygen were absorbed per g of liver per hour in the normal rats, and 0 to 36μl of oxygen were absorbed in the DAB fed rats. The addition of flavin adenine dinucleotide (FAD) into the homogenate never improved the activity of this enzyme in the liver of rats fed DAB.

This fact indicates that the protein part of this enzyme is diminished by DAB feeding.

Table 1. D-Amino Acid Oxidase in the Liver of Normal and DAB-fed Rats.
(Expressed by μl. of O_2 absorbed/g of liver/hour)

Exp. No.	Normal Rat	Exp. No.	DAB fed Rat
1	40	10	0
2	48	11	0
3	51	12	0
4	51	13	18.5
5	84	14	33
6	93	15	36

2) *Xanthine Oxidase*

Xanthine oxidase activity was denoted by μM of xanthine disappeared per g of liver per hour. No change in activity of this enzyme calculated from the initial velocity was observed as shown in Table 2.

Table 2. Xanthine Oxidase in the Liver of Normal and DAB-fed Rats.
(Expressed by μM of Xanthine disappeared/g of liver/hour)

Exp. No.	Normal Rat	Exp. No.	DAB fed Rat
17	49	21	50
18	55	22	50
19	55	23	50
20	60	24	55
		25	66

There exists, however, a remarkable difference between the enzyme activities of normal and of DAB fed rats. Although the xanthine oxidase in the homogenate prepared from normal rat liver never changed the reaction velocity up to 120 minutes,

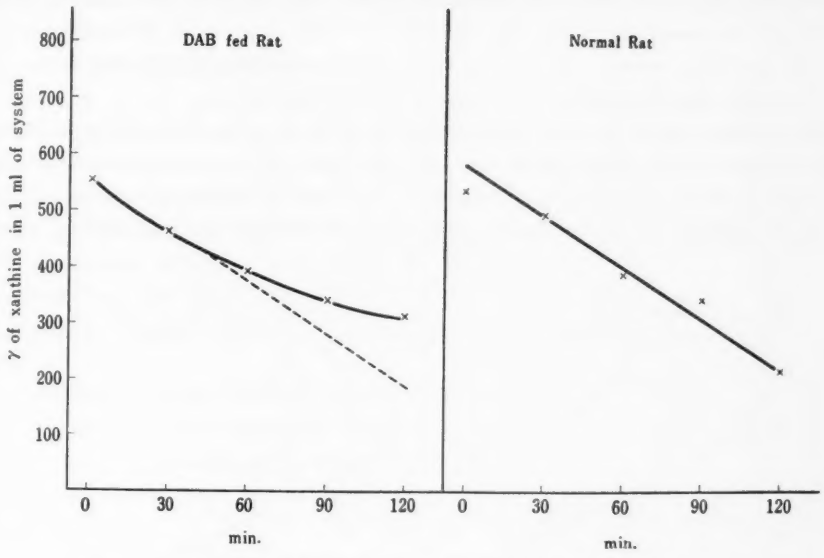

Fig. 1 Xanthine Oxidase Activity in Normal and DAB fed Rat Liver.

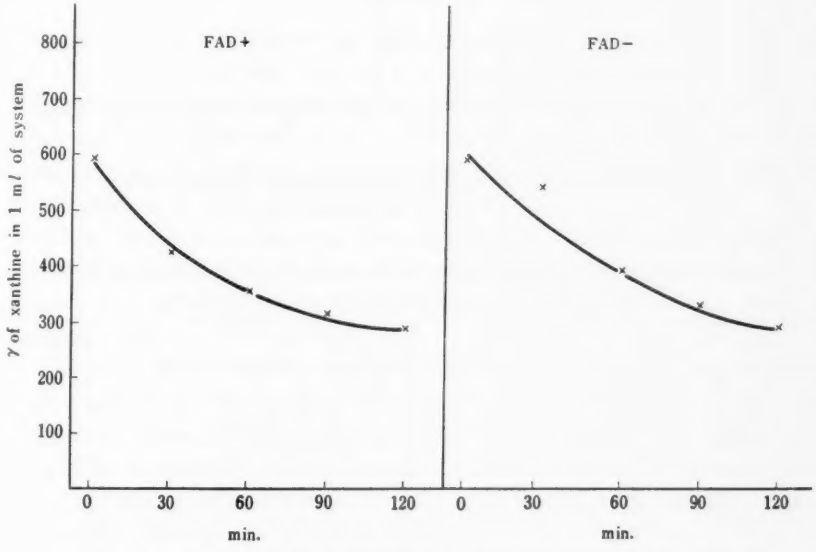

Fig. 2 Effect of FAD on the Xanthine Oxidase in the Liver of Rat fed DAB.

the enzyme activity in the homogenate prepared from the liver of rats fed DAB gradually decreased after 30 minutes (Fig. 1). The addition of FAD did neither elevate the initial velocity nor prevent from this deterioration phenomenon shown by the xanthine oxidase in the liver of rats fed DAB (Fig. 2).

These facts suggest that the shape of protein molecule around the active center of this enzyme has not been altered, but the other part of enzyme protein has been damaged to some extent by DAB feeding. But there is another possibility that the protective protein for the xanthine oxidase may be damaged by DAB feeding. DAB

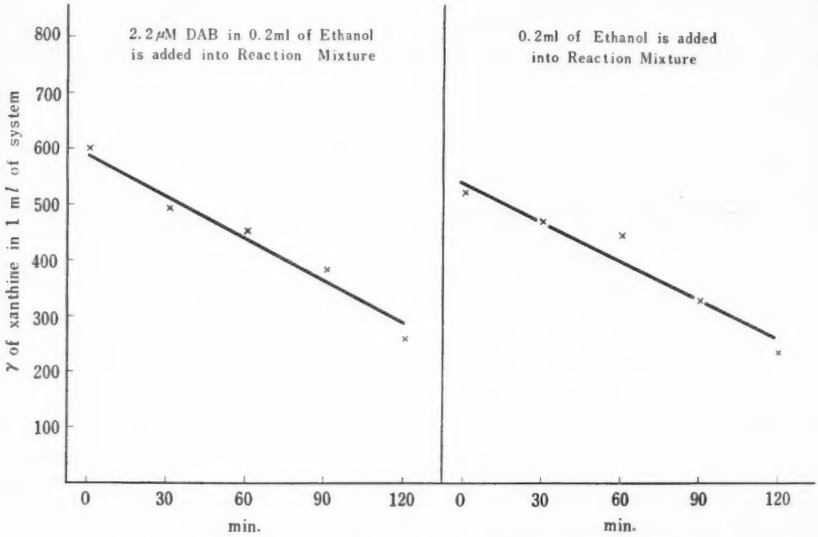

Fig. 3 In vitro Effect of DAB on the Activity of Xanthine Oxidase in Normal Rat Liver.

Table 3. Succinic Dehydrogenase in the Liver of Normal and DAB-fed Rats. (Expressed by μl of O_2 absorbed/g of liver/5 min.)

Exp. No.	Normal Rat	Exp. No.	DAB fed Rat
26	275	31	0
27	290	32	22
28	305	33	110
29	325	34	157
30	330	35	205
		36	250
		37	265
		38	275

had no influence on the xanthine oxidase activity in the normal rat liver homogenate *in vitro* (Fig. 3).

3) *Succinic Dehydrogenase*

The activity of succinic dehydrogenase in the liver homogenate was expressed by μl of oxygen uptake per g of liver per five minutes. As shown in Table 3, 275 to 300 and 0 to 275μl were found in the normal and DAB fed rat livers, respectively.

This means a decrease of the enzyme activity in the liver of DAB fed rats, but the enzyme activity of DAB affected liver can be almost completely restored by addition of FAD in vitro (Fig. 4).

These facts indicate that the apoenzyme of succinic dehydrogenase is kept intact and the prosthetic group of this enzyme is ejected from the enzyme by DAB feeding.

DAB has also no effect on this enzyme activity in vitro.

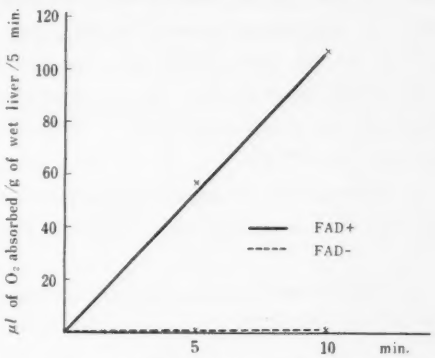

Fig. 4 Effect of FAD on the Succinic Dehydrogenase in the Liver of Rat fed DAB.

Discussion

Recently, Deij (7) has studied the effect of riboflavin-deficient diet on the activity of flavin enzymes in rat liver, and the most serious decrease was found in the activity of D-amino acid oxidase. No decrease in xanthine oxidase was observed. Although full information of Deij's work is not available at present, the decreased activities of flavin enzymes caused by flavin-deficient diet may be considered to be restored by the replenishment of flavin coenzymes in vitro.

When the rats were fed on DAB, they might develop a condition of flavin deficiency. So, some of the present observations seem to be in accord with Deij's finding. But still there may be some fundamental differences such as denaturation of enzyme proteins.

Present experiments showed that the different flavin enzymes are affected in different manners by DAB feeding. Among flavoproteins tested, D-amino acid oxidase was the only enzyme of which apoprotein was seriously damaged in DAB feeding. Although the physiological role of D-amino acid oxidase in the animal body remains obscure, the facts discussed above may imply this enzyme to be playing a role as a flavin storaged protein.

Studies on the effect of DAB on the other flavin enzymes are now being carried on.

Summary

1) Rats were fed on DAB for about one month, and the activities of some flavin enzymes in the liver were tested.

2) D-Amino acid oxidase was damaged seriously by DAB feeding, and its activity was never improved by the *in vitro* addition of FAD.

3) The initial reaction velocity of xanthine oxidase prepared from DAB-affected rats was not reduced when compared with the enzyme from the normal, but the velocity of the DAB-affected enzyme gradually dropped off during incubation. This deterioration phenomenon was not observed in the normal enzyme. The addition of FAD to the reaction mixture of DAB-affected enzyme had no improving effect on the enzyme activity.

4) Succinic dehydrogenase activity was decreased by DAB feeding, but the addition of FAD to the reaction mixture almost completely restored its activity.

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STUDIES ON THE MECHANISM OF LIVER CARCINOGENESIS BY CERTAIN AMINOAZO DYES. III. EFFECT OF PARAFORMALDEHYDE, DIMETHYL-*p*-PHENYLENE DIAMINE AND DISULFIRAM UPON THE CARCINOGENIC POTENCY AS WELL AS THE PROTEIN BINDING OF AMINOAZO DYES AND THE CARCINOGENICITY OF DIMETHYLAMINOAZOXY-BENZENE-N-OXIDE.

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INTRODUCTION

As one of the causative steps of the chemical carcinogenesis induced by the aminoazo dyes, the Millers have postulated a protein deletion theory (1) (2) based on the finding of the formation of the azo dye bound proteins in the precancerous liver and of the deletion of the corresponding protein fraction in the azo dye induced hepatoma. Sorof et al. (3) (4) added another observation that an electrophoretical protein fraction, "slow h_2", which contains most of the azo dye bound proteins, rather increased in the precancerous liver, although it was depleted in the tumor.

The chemical nature of the binding between azo dyes and proteins has been studied by Terayama et al. (5) (6) (7) (8), using the highly purified polar dye preparations. They assumed tentatively that the binding between the azo dye and the protein consisted of a methylene bridge. The origin of the methylene bridge was considered to be primarily from N-methyl of the dye and partly from the endogenous C_1 donors. The metabolism of N-methyl group, therefore, seems to be important in elucidating the carcinogenic mechanism of the dyes. It is very interesting to see that the enzyme system which is responsible for the oxidative N-demethylation as well as other important metabolism of aminoazo dyes such as hydroxylation and azo reduction is located in the microsomes (10) (11) and that the dye-protein binding takes place also in the microsome fraction at the beginning (12) (13) (14). The polar dyes derived from N-methyl-C^{14}-labeled MAB are highly radioactive regardless of the nature of the amino group (15). The fact that a polar dye fraction having the primary amino group has

the radioactivity indicates that the methyl group of MAB administered is incorporated in some way into the polar dye molecule at a position other than the amino group of the azo dye moiety.

In this paper, the experiments will be reported which have been carried out in order to investigate the significance of the N-methyl group metabolism in the chemical carcinogenesis by the azo dyes.

The effect of the oral administration of paraformaldehyde upon the azo dye-protein binding was investigated, using aminoazobenzene (AB) and dimethylaminoazobenzene (DAB). Nochbin (Disulfiram) (16) was investigated on its effect upon the polar dye formation when DAB was administered, considering its strong antialdehyde dehydrogenase activity which might facilitate the dye-protein binding. Azoxy-DAB-oxide (4-Dimethylamino-β-azoxybenzene-4-N-oxide) was studied because the N-oxide was assumed to be one of possible intermediates (17) in the oxidative N-demethylation of DAB. A series of experiments was also carried out with the idea that the addition of formaldehyde or N,N-dimethyl-*p*-phenylene diamine to the AB-containing diet might increase both the carcinogenic potency and the dye-protein binding. The non-carcinogenicity of dimethyl-*p*-phenylene diamine had been shown (18).

Experimental

Treatment of the Animals: Albino rats weighing 120–130g were fed ad libitum. The diet was rice as the basal component, which was mixed with an azo dye and other ingredients to be tested. The concentration of the dye (AB or DAB) was constant at 0.06%. Propylene glycol instead of olive oil was used throughout these experiments as a solvent for the azo dyes without any special reason. From time to time, usually one rat at a time except in the case specially noted was killed by decapitation, and liver was removed immediately after perfused in situ with the physiological saline solution. The liver thus treated was used for the estimation of the protein bound dye.

Estimation of the Polar Dyes: The dry defatted liver powder was prepared by washing the liver homogenate thrice with alcohol, twice with acetone and with ether successively. The liver powder was suspended in the M/10 sodium acetate (pH 7.0) (about 1–5% suspension) and hydrolyzed with the purified protease prepared from *Streptomyces griceus* culture as described in the preceding paper (5) (7) (8) (9), where the detail of the technique including the following extraction and measurement of the polar dyes are also described. The amounts of the polar dyes are expressed in this paper in term of 3-Me-AB in the case of AB administration, and in term of DAB in the case of DAB administration.

Preparation of Azoxy-DAB-N-oxide (20): 37g of DAB (one equivalent) was dissolved in a small amount of benzene and the benzene solution of perbenzoic acid (two equivalents) was added under constant stirring. The crystalls appeared instantly.

After stored in cold for two hours, the crystalls were filtered, washed with a small volume of benzene and recrystallized from benzene. Yield : about 40g. mp. 135°-137°C.

Results and Discussion

The Amounts of Protein bound Dye in the Time Course of the Administration of AB, AB plus Paraformaldehyde, and AB plus Dimethyl-p-phenylene diamine : The amounts of the protein bound dye are shown in Table 1.

Table 1.

The amounts of the protein bound dyes in the time course of the administration of AB, AB+PFA and AB+DPD.

(PFA: paraformaldehyde 0.06%; DPD: dimethyl-p-phemylene diamine 0.01%; AB: aminoazobenzene 0.06%)

Ingredients	Feeding Interval (days)	Liver wt. (g)	Amount of protein bound dye	
			measured (γ)	cald. per 10g liver (γ)
AB	7	7.6	2.2	3.0
	17	7.4	1.7	2.3
	22	6.0	3.9	6.5
	35	6.4	4.9	7.7
	53	6.8	3.6	5.3
AB+PFA	2	6.3	3.9	6.2
	7	5.0	2.2	4.4
	11	6.8	2.9	4.2
	17	6.8	2.0	3.0
	22	7.9	3.9	5.0
	29	7.0	6.4	9.1
	35	8.5	7.0	8.2
	44	7.6	2.3	3.0
	53	8.2	8.2	10.0
	60	8.1	7.6	9.4
	249	22.2 (3 rats)	3.5	1.6
AB+DPD	6	7.9	5.6	7.1
	13	11.4	7.1	6.2
	17	7.0	5.2	7.4
	31	7.0	4.2	6.0
	181	68.7 (7 rats)	8.9	1.3

As shown in Table 1, the effect of the addition of PFA or DPD in the diet containing AB upon the amounts of the polar dye formed appears not to be so significant, though it seems that the polar dye formation in the first several days in

Table 2.

The amounts of the protein bound dyes in the time course of the administration of DAB, azoxy-DAB-N-oxide, DAB+PFA and DAB+Disulfiram.

(DAB: 0.06%, Azoxy-DAB-N-oxide; 0.06%, PFA: 0.06%, Disulfiram: 0.3%)

Ingredients	Feeding Interval (days)	Liver Wt. (g)	Amounts of Protein bound Dyes	
			Observed (γ)	per 10g liver (γ)
DAB	3	7.3	4.8	6.6
	10	6.6	9.0	13.6
	17	6.6	14.0	21.2
	19	6.2	3.5	5.6
	21	7.0	5.9	8.4
	24	7.0	20.4	29.1
	33	6.4	14.2	22.1
	35	4.6	9.7	21.1
DAB+PFA	9	7.1	12.0	16.9
	12	6.9	13.2	19.1
	22	8.5	17.8	20.9
	27	9.0	5.8	6.5
	〃	8.2	7.8	9.5
	〃	9.1	5.0	5.5
DAB+ Disulfiram	5	10.5	2.9	2,7
	10	5.6	2.1	3.7
	14	7.8	4.6	5.9
	20	6.7	5.1	7.6
	25	10.3	4.9	4.8
	32	11.0	16.1	14.6
	38	8.2	15.5	23.8
	47	9.4	17.5	18.6
	56	10.2	10.4	10.2
	63	11.8	11.0	9.3
	70	12,0	2.7	2.2
Azoxy-DAB-N-oxide	18	15.5 (3 rats)	2.5	1.6
	19	5.3	4.5	8.5
	28	31.0 (3 rats)	13.9	4.5
	39	19.3 (3 rats)	14.6	7.6
	48	12.4 (2 rats)	12.5	11.0
	60	15.2 (2 rats)	22.4	14.6
	83	18.5 (2 rats)	7.9	4.2
	102	18.1 (2 rats)	50.2	27.0
	105	40.0 (5 rats)	34.3	8.6
	107	21.8 (2 rats)	6.5	3.1

the cases of AB+PFA and AB+DPD administrations is a little larger than in the case of AB only. The results however are still inconclusive because of the rather great individual fluctuation. The level of the polar dye formation is only about a third of the amount with DAB administration.

The Amounts of the Protein bound Dyes in the Time Course of the Administration of DAB, Azoxy-DAB-N-oxide, DAB+PFA and DAB+Disulfiram: The amounts of the polar dyes in the time course of the administration of DAB, Azoxy-DAB-N-oxide, DAB+PFA and DAB+Disulfiram are listed in Table 2.

As shown in Table 2, the addition of PFA to DAB does not strengthen the incorporation of the dye into the protein as in the case of AB+PFA. The retardation of the polar dye formation by the addition of disulfiram was clearly observed. It was found that the disulfiram inhibits not only the aldehyde dehydrogenase but also the metabolism of the dye, including the oxidative N-demethylation of 3-Me-MAB (21).

It is very interesting to see that the polar dye formation by the administration of the azoxy-DAB-N-oxide is very slow compared with DAB. The reason is still obscure. Considering the higher solubility of azoxy-DAB-N-oxide in water, the high excretion rate of the N-oxide may at least partly be responsible for the observed effect.

The chemical nature of the polar dye derived from azoxy-DAB-N-oxide was shown to be a little different from the DAB polar dye as will be described in the following section.

The Polar Dye derived from Azoxy-DAB-N-oxide: The livers of rats fed on the rice containing azoxy-DAB-N-oxide (0.06%) were pooled and the polar dye was prepared from them. After the hydrolysis of the liver, the hydrolysate was washed with benzene to eliminate a small amount of the nonpolar dye. The polar dye was extracted with n-butanol and the butanol was evaporated under the reduced pressure. The residue was dissolved in 2N HCl. The pink HCl solution was washed with ethyl acetate. The HCl layer separated from ethyl acetate was warmed in a water bath under the reduced pressure to eliminate the dissolved ethyl acetate. The solution was then subjected to the IRC-50 (H type) column chromatography. The dye was absorbed at the top of the column. After washed with 2N HCl, water and then with 30% alcohol, the dye was eluted with 2N ammonia. The polar dye thus prepared was shown to be a mixture and could be further fractionated into a few components with IRC-50 (H type) column chromatography when the dye was eluted with 60% alcohol or 50% alcohol containing 2N HCl. It was clear from the HNO_2 test that the dye consisted of at least two types of aminoazobenzenes, one the primary amino type and the other the secondary amino type.

Comparing the polardye derived from azoxy-DAB-N-oxide with the polar dye from DAB, the most distinguished difference was that the former contains the rather acid labile polar dyes. When the polar dye from azoxy-DAB-N-oxide was refluxed in 2N

HCl for two and a half hours, 30% or more nonpolar dye was shown to be liberated. In N-H_2SO_4 the decomposition was about 60% after 4.5 hours of reflux. The dye thus liberated was mostly MAB, identified by the spectral and HNO_2 examinations. The polar dye from DAB treated under the similar conditions did not yield any appreciable amount of the nonpolar dye.

When the polar dye dissolved in HCl solution was stored at room temperature for a month or so, the pink solution gradually turned to violet and finally to brown. This color could be separated from the pink color with the aid of the IRC-50 (H type) chromatography, because it comes into the flow through in 2N HCl. The polar dye from the azoxy-DAB-N-oxide is clearly a mixture of acid labile and acid stable dyes.

Polar Dye from DAB+PFA : The liver of rats fed DAB+PFA was treated as described in the preceding paper, and the polar dye was isolated. The polar dye thus prepared has an absorption max. at 520mμ in 2N HCl and 50% alcohol solution, and shows the presence of the secondary amino type dye in predominance, judged

Table 3.
The carcinogenicity of AB+PFA, AB+DPD, Azoxy-DAB-N-oxide compared with DAB.

Carcinogens	Body wt. (g)	Liver wt. (g)	Liver findings	Feeding Time (days)
DAB (0.06%)	110	11.9	+ pea sized tumors	183 sacrif.
	210	13.5	+	〃 〃
	248	12.5	− granular hyperplasia	〃 〃
	120	11.4	+	〃 〃
	204	16.1	+	〃 〃
	232	12.9	± very small tumor	〃 〃
AB (0.06%) PFA (0.06%)	180	7.5	− No cirrhosis	249 sacrif.
	180	7.2	− 〃	〃 〃
	180	7.5	− 〃	〃 〃
AB (0.06%) DPD (0.01%)	270	7.8	− a little rough liver surface	181 sacrif.
	260	12.0	− 〃	〃 〃
	210	9.2	− 〃	〃 〃
	230	9.2	− 〃	〃 〃
	230	11.5	− 〃	〃 〃
	240	10.5	− 〃	〃 〃
	188	8.9	− 〃	〃 〃
Azoxy-DAB-N-oxide (0.06%)	——	——	− No pathological lesion	118 sacrif.
	138	6.5	− precirrhotic	178 dead
	180	33.7	+	314 dead

from the reaction to HNO_2 test. The polar dye formed upon feeding only DAB was shown to consist mostly of the primary amino type polar dye and a small fraction of the secondary type polar dye. The addition of paraformaldehyde seems to favor the formation of the secondary amino type polar dye.

Carcinogenicity of AB+PFA, and AB+DPD and Azoxy-DAB-N-oxide: The carcinogenicity of DAB (0.06%), AB (0.06%)+PFA (0.06%), AB (0.06%)+DPD (0.01 %), and azoxy-DAB-N-oxide (0.06%) was compared after a long term of the feeding. The rats weighing 120 to 130g at the beginning of the experiment were used. The results are shown in Table 3.

It has been reported (22) that AB could hardly be methylated in vivo although the mutual transformation between MAB and DAB is very easy (DAB$\rightleftharpoons$MAB$\longrightarrow$AB). The apparently peculiar irreversibility between MAB and AB might be due to the lack of a methyl donor. It was therefore expected that the addition of formaldehyde or N, N-dimethylphenylenediamine might facilitate the N-methylation of AB in vivo and henceforce generate the carcinogenicity. Contrary to the above expectation, however, the addition of paraformaldehyde or N,N-dimethyl-p-phenylenediamine to the diet containing AB under the experimental condition described above does not seem to favor either the increased formation of the polar dye or shortening the induction period of the carcinogenesis.

This fact might be interpreted as indicating that AB is too quickly metabolized in vivo to be methylated and the dye protein binding ia an instantanous reaction in the course of the N-methyl metabolism (oxidative demethylation), perhaps in statu nascendi.

Both the carcinogenicity and the polar dye formation by azoxy-DAB-N-oxide was greatly reduced compared with the original DAB. It still remains unsolved that the N-oxide form could be an intermediate in the oxidative N-demethylation of DAB in vivo. Anyway the metabolism of azoxy-DAB-N-oxide is greatly altered, thus reducing the rate of the polar dye formation as well as the rate of the carcinogenesis compered with DAB.

The effect of disulfiram on the polar dye formation may also be ascribed to the reduced metabolic activity of the rat liver against DAB.

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EFFECT OF TRANSPLANTED TUMOUR ON THE PYRIDINE NUCLEOTIDE SYNTHESIS IN THE MOUSE LIVER*

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It is well known that the level of pyridine nucleotide (PN) is generally very low in actively growing tissues such as tumour or foetal tissues (1-8). It has been suggested that the decrease of PN level in these tissues may be caused by a diminished rate of the PN synthesis rather than by an increase of its destruction (8). On the other hand, a marked increase of the PN content has been observed in the mouse liver following the injection of nicotinamide which is acting thereby as a precursor substance of PN (9, 10). In a previous paper, the author has reported that the intrasplenic implantation of a nicotinamide pellet is very effective in elevating the PN level of normal and regenerating rat liver and that the numbers of mitoses are thereby greatly reduced (11). It has also been reported that the decrease of the PN level is a general occurrence in livers of the tumour-bearing animals, indicating that there may be a sort of hormonal intervention (12). It is possible that the adrenal, especially adrenocortical hormones are involved in such hormonal intervention, since the presence of a tumour may act as a stressor. In the present study, the capability of PN synthesis in livers of tumour-bearing mice was tested by injecting nicotinamide, and furthermore the effect of cortisone acetate on the PN level in these mice was examined.

Materials and Methods

Male albino mice weighing from 15 to 20 g were used as material throughout the experiments, and Ehrlich ascites tumour was exclusively used, being intraperitoneally transplanted. One or two weeks after the transplantation of the tumour, animals were sacrificed and examined for the PN level, since tumour cells undergo a rapid growth during this period.

Nicotinamide (1,000 mg/kg of body weight) was administered intraperitoneally or subcutaneously 10 hours before the sacrifice. In experiments in which cortisone acetate was used, 100 micrograms of it were injected subcutaneously for two days, the last injection being performed 15 hours before the sacrifice.

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The PN content of the liver and tumour cell was determined by the method of Feigelson, Williams and Elvehjem (13) with a slight modification. Animals were decapitated and the livers were quickly removed and about 300 mg of the tissues were weighed on a torsion balance before the determination of PN. The details of the procedure have already been described in the previous report (11), but in the present experiment, 100 mg of acid-washed and dried Norit SX 30 were used to adsorb PN.

Another piece of the liver weighing about 200 mg was used for the determination of protein N, this beeing done by the micro-Kjeldahl method, and the values obtained served as the basis of calculation of PN level. The PN was calculated as diphopyridine nucleotide (DPN).

For the determination of PN in tumour cells, the ascites was quickly pipetted out before the removal of the liver, and transferred into a centrifuge tube which had been placed in an ice-bath. Then, about 2 volumes of ice-cold isotonic NaCl solution were added and centrifuged at 500 r. p. m. for 5 minutes to separate the red blood cells. To the precipitated tumour cells were added about 3 volumes of ice-cold isotonic NaCl solution and centrifuged at 3,500 r. p. m. for 2 minutes to remove the ascites. About 2 ml of washed tumour cells were suspended in an ice-cold isotonic NaCl solution, and the volume was adjusted to 10 ml. Each 2 ml of the suspension were used for the PN and protein N determination respectively. For the extraction of PN, 2 ml of the suspension were decanted into a glass homogenizer tube containing 2 ml of ice-cold 10 per cent trichloroacetic acid and 0.5 ml of 30 per cent hydrogen peroxide, then homogenized for a minute. After the addition of 6 ml of deionized water and 1 ml of 2 per cent trichloroacetic acid, it was again homogenized for a minute. The other procedure was the same as in case of the liver.

Results

The changes in the PN content of livers of normal and ascites-tumour-bearing mice following the administration of nicotinamide are represented in Table 1. It will be seen that a definite increase of the PN level is brought about in the livers of both normal and tumour-bearing animals by the administration of nicotinamide, although the increase is not very marked in the case of tumour-bearing mice as compared with the normal. In livers of normal mice which received no injection, 1.97 mg of PN per 100 mg of protein N was found on an average and 10 hours after the subcutaneous injection of nicotinamide, the PN was found to rise to 14.0 mg per 100 mg of protein N. It is to be noted that during a week or two after the transplantation of Ehrlich ascites tumour, the average PN value for the liver decreased to 1.35 mg per 100 mg of protein N from the value of 1.97 mg, indicating that the PN level of the liver of the tumour-bearing animal is about 70 per cent of that of

the liver of intact normal animal. However, 10 hours after the intraperitoneal or subcutaneous injection of nicotinamide, the PN content of the liver of tumour-bearing animals was found to increase to 7.05 or 7.40 mg per 100 mg of protein N.

Table 1. The changes of the PN content in livers of normal and tumour-bearing mice 10 hours after the injection of nicotinamide (1, 000 mg/kg of body weight.) The values represent mg of PN/100 mg of protein N. Changes in the PN content of the tumour cells under the same conditions are also given.

Animal	Treatment					
	No Treatment		Intraperitoneal Injection of Nicotinamide		Subcutaneous Injection of Nicotinamide	
	Liver	Tumour	Liver	Tumour	Liver	Tumour
Normal Mouse	1.21				16.2	
	2.29				15.5	
	1.41				13.3	
	2.23				13.1	
	2.73				11.7	
(Average)	1.97				14.0	
Tumour-bearing Mouse	1.22	—	9.61	2.75	8.99	4.24
	1.79	1.75	5.84	2.81	4.80	2.72
	1.03	1.89	5.13	3.67	9.61	2.75
	1.13	1.73	7.62	2.13	6.00	3.41
	1.60	2.52			7.59	3.35
(Average)	1.35	1.97	7.05	2.84	7.40	3.29

The changes in the PN content of tumour cells as infiuenced by nicotinamide injection are also shown in Table 1. From this Table it is obvious that the PN content of the tumour cells differs little from that of livers of intact normal mouse, and the PN level of the tumour itself increases to 2.84 and 3.29 mg per 100 mg of protein N after the intraperitoneal and subcutaneus injection of nicotinamide, respectively. From these results, it is also clear that there is no difference in the effect produced on the PN content of the liver or tumour cells between the intraperitoneal and subcutaneous injection of nicotinamide. Therefore, only the subcutaneous injection was employed in the next experiments.

After having confirmed that nicotinamide is very effective in accelerating the synthesis of PN whether in livers of normal and tumour-bearing animals or tumour itself, the following experiments were performed in order to decide whether the hormones of the adrenal have some effect on the PN level of these tissues.

Cortisone acetate was subcutaneously injected into mice and the PN levels of livers and tumour cells were examined. The results are shown in Table 2. It was found

Table 2. The effect of cortisone acetate on the changes in the PN content of livers of normal and tumour-bearing mice following the subcutaneous injection of nicotinamide. The values represent mg of PN/100 mg of protein N. The effect on the PN content of tumour cells is also given.

	Treatment							
	No Treatment		Nicotinamide Alone		Cortisone Acetate Alone		Cortisone Acetate & Nicotinamide	
	Liver	Tumour	Liver	Tumour	Liver	Tumour	Liver	Tumour
Normal Mouse	See Table 1.		See Table 1.		1.44		9.89	
					1.23		11.8	
					1.22		8.23	
					1.47		6.92	
							5.94	
(Average)	1.97		14.0		1.34		8.56	
Tumour-bearing Mouse	See Table. 1		See Table 1.		2.13	2.18	3.63	1.41
					0.88	1.95	7.37	2.73
					1.42	2.45	5.93	3.67
					2.03	2.51		
					1.09	2.19		
(Average)	1.35	1.97	7.05	2.84	1.51	2.26	5.64	2.60

that in livers of normal mice, the PN content was lowered to 1.34 mg per 100 mg of protein N after the administration of cortisone acetate, and this level is the same as that in the liver of tumour-bearing animal. When cortisone acetate was administered to normal mouse prior to the injection of nicotinamide, the PN content of the liver increased to 8.56 mg per 100 mg of protein N. This increase was about half that caused by nicotinamide alone and almost identical to that observed in the liver of tumour-bearing mouse after the administration of nicotinamide. These results may be taken to indicate that the depression of the PN synthesis in livers of tumour-bearing mice is brought about by the intervention of some such adrenal hormone which is liberated in excess in these animals. However, it is to be noted that the livers of tumour-bearing mice and ascites tumour cells were not affected so strongly by cortisone acetate as in the case of normal liver. Especially, in the case of tumour cells, the injection of cortisone together with nicotinamide caused an increase of the PN level nearly to that found after the injection of nicotinamide alone. In the case of the liver of tumour-bearing mouse, only a slight reduction of PN synthesis was observed when cortisone acetate was injected combined with nicotinamide.

It may be concluded therefore that cortisone acetate exerts a definite influence on the synthesis of the PN in the liver of normal animal, depressing the PN level to

that of the liver of the tumour-bearing animal and that the decreased PN level of the liver of the tumour-bearing animal or tumour itself is no longer influenced by the cortisone administration.

Discussion

In was previously reported by Waravdekar (12) that the livers of animals bearing various forms of tumour showed a decreased capacity for DPN synthesis, and that following the surgical removal of the tumour the DPN synthetic activity returned to normal. He also suggested that some hormonal intervention is likely in the reduction of DPN synthesis in the livers of tumour-bearing animals.

On the other hand, it is well known that the liver catalase activity of tumour-bearing animals is generally depressed, and Nakahara and his colleagues (14) have reported that a fraction extracted from the tumour tissues and named Toxohormone, invariably causes a marked decrease in the catalase activity in the mouse liver *in vivo*. Furthermore, it was reported by Ono and Tomaru (15) that the DPN synthesis in mouse liver was also depressed by this Toxohormone both *in vivo* and *in vitro*, although it was less effective *in vitro* than *in vivo*. In this connection, the report of Utsugi (16) is very interesting. He has shown that the characteristic depression of catalase activity no longer occurs in tumour-bearing rats and mice, when they are hypophysectomized and kept alive, and concluded that a certain pituitary hormone, presumably the growth hormone, plays a definite role in the depression of liver catalase activity which takes place in livers of these tumour-bearing animals. From these findings it is likely that the adrenal plays some part in the decrease of PN content in the liver of tomour-bearing animal through the intervention of the pituitary.

In the present study, a marked reduction of the capability of PN synthesis in the liver of tumour-bearing and cortisone-treated mouse has been demonstrated. The increase of PN which is brought about by the administration of nicotinamide in the liver of tumour-bearing mouse is only half that observed in livers of normals. If cortisone acetate is administered prior to the injection of nicotinamide, there occurs a marked reduction of PN synthesis in the case of normal liver, the PN level becoming very similar to that in the liver of tumour-bearing animal. However, the administration of cortisone acetate produces no longer any marked effect upon the already reduced synthesis of PN in the liver of tumour-bearing animal. These results seem to indicate that there is the particiption of the adrenal hormone in the reduction of PN synthesis in the liver of tumour-bearing animal, although it is not certain whether the adrenal hormone is acting directly or through the pituitary.

In the case of the Ehrlich ascites tumour cells, the level of PN was relatively high. However, their activity of PN synthesis as influenced by the administration of nicotin-

amide was very low. Even if the intraperitoneal injection of nicotinamide was employed, the PN level of tumour cells was no more than 150 per cent of that observed in the animals received no injection. Also PN synthesis in tumour cells was not affected by cortisone acetate.

Lastly, it may be mentioned that there is some evidence that the lowered PN content of the tumour or other growing tissues is associated with the rate of cell proliferation (11, 17, 18). Morton (17) and Sahsrabudhe (18) are of the opinion that the lowered PN level causes the cell to divide by some 'feed-back' mechanism in connection with energy metabolism. In these discussions, it has been assumed that the respiratory activity is low and glycolytic activity high in tumour tissues. However, Weinhouse (19) has shown that the low respiratory activity is only found in some specific types of tumour such as Ehrlich ascites and it is not always found in all tumours. From these facts, it is suggested that the energy metabolism in the tumour tissues is sufficiently maintained by such a decreased activity of PN synthesis as found in these tissues, whereas the relatively high level of PN and the high activity of its synthesis as observed in normal liver cell must be utilized for the functional activity such as secretion or detoxication and so on. Therefore, it will be interesting to examine closely the functional activity of the liver of tumour-bearing animals, in which the PN level has been greatly lowered.

Summary

1. The capability of PN synthesis as influenced by nicotinamide administration is very low in the Ehrlich ascites tumour cell and the liver of its host mouse, as compared with that of normal mouse liver. In the case of the liver of tumour-bearing mouse, the increase of PN after the administration of nicotinamide is half that observed in normal mouse liver.

2. After the administration of cortisone acetate, the activity of PN synthesis in normal mouse liver always decreases to the level of tumour-bearing liver, suggesting some such hormone may be acting in the tumour-bearing animal.

3. However, the already reduced activity of PN synthesis in the liver of mouse bearing ascites tumour and tumour cell itself is hardly affected by the administration of cortisone acetate.

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EFFECT OF IRON ADMINISTRATION ON THE FREE PROTOPORPHYRIN LEVELS IN ERYTHROCYTE AND LIVER OF TUMOR-BEARING RATS.*

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There have been accumulated the evidences to indicate the disturbance of iron metabolism in tumor-bearing animals and cancer patients. The marked liver catalase depression, anemia (1), decreased iron levels in serum (2, 3, 4, 5, 6) and low liver ferritin content (7) are the general symptoms in tumor-bearing hosts. These symptoms of disturbed iron metabolism have been reproduced in normal mice and rats by the injection of toxohormone isolated from tumor tissues (6, 8, 9), and, moreover, the effect of toxohormone on liver catalase can be partially overcome by simultaneous supplement of iron, as reported by Fukuoka and Nakahara (10).

The accumulation of free protoporphyrin in blood cell and liver of tumor-bearing rat, as already demonstrated by us (11, 12), may also be considered to indicate the retardation of the rate of iron insertion into the protoporphyrin ring to form heme, in the presence of growing tumor.

If the assumption is correct that there is a state of iron deficiency in tumor-bearing animals, these symptoms describe above would be expected to be overcome to variable extents by the administration of iron in high doses to the tumor-bearing animals, according to the degree of the dependency of each symptom on iron metabolism. From this point of view we carried out the experiments on the influences of iron administration on the free protoporphyrin levels in blood cell and liver of tumor-bearing rats, and on the hemoglobin and liver catalase activity also.

Material and Methods

The general experimental conditions were the same as those of our previous reports (11, 12). Male albino rats (Saitama strain) were transplanted with Rhodamine fibrosarcoma and separated into two groups and kept in brass wire cages. One of the groups was restricted in the iron supply by feeding polished rice and fresh vegetables only, the other groups was fed with the same diet supplemented with reduced iron at the rate of 2 mg/head/day. At the same time, two control groups with iron restricted and iron supplied diets were started. 14-27 days after transplantation, the

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rats were sacrificed with the appropriate controls and hemoglobin, reticulocytes, liver catalase activity and free protoporphyrins in blood cell and liver were determined. All the determination procedures were the same as those described in our previous reports (11, 12). To exclude the misinterpretation caused by the variation in tumor weights between iron restricted and supplied groups, only the animals with tumors weighing about 20% of body weight were used in this experiments. And the total iron of liver was assayed to ascertain the effect of iron restriction and load.

Results

In Table 1 is presented a summary of the mean values of hematocrit, hemoglobin, reticulocytes, erythrocyte protoporphyrin, liver total iron, liver catalase and liver free protoporphyrin of each group, which consisted of 13–15 rats.

Table 1. Effects of Iron Administration upon the Rats Bearing Rhodamine Fibrosarcoma.

		Normal rats		Sarcoma-bearing rats	
		Iron restricted	Iron fed	Iron restricted	Iorn fed
	Number of rats	14	15	13	14
	Body weight (g)	207±3.92	189±7	195±9	200±10
	Tumor weight (g)	—	—	38.3±5	36.7±4
Blood	Hematocrit (%)	50.4±3.92	52.3±2.45	37.7±7.49	45.1±4.97
Blood	Hemoglobin (g/dl)	14.1±0.95	14.6±0.67	9.4±2.14	11.3±1.06
Blood	Reticulocyte (%)	17.1±8	25.1±9	171±25	208±34
Blood	Free protoporphyrin (μg/dl of blood cells)	65.9±24.5	57.4±9.6	154.2±41.5	82.7±11.5
Liver	Total iron (μg/g)	71.6±8.8	144.2±45	51.6±15.2	151.0±41
Liver	Catalase activity (k/min.)	0.200±0.034	0.241±0.028	0.116±0.055	0.149±0.033
Liver	Free protoporphyrin (μg/dg)	38.1±9.3	27.9±8.4	50.8±24.1	24.0±7.8
Liver	Extinction at 493 mμ of protoporphyrin fraction	0.051±0.003	0·041±0.004	0.104±0.007	0.050±0.003

The figures in the table represent the average of each determination of each group and its 90% reliability limit.

The reduced iron supplied in the diet was extraordinarily high (2 mg/day) and it elevated the total iron level in the liver of tumor-bearing rats to as far as 150 μg/g. On the other hand, the total iron level in the liver of the iron restricted ones was only 51.6μg in tumor-bearings and 71.6μg/g in normal rats, and in these cases the deprivation of iron from the liver of tumor bearers was confirmed as was reported in the previous paper (7).

While in the control groups without tumor, the iron supplement showed scarecely any effect on hematocrit value and hemoglobin content in blood, it could prevent the anemia of tumor-bearers though not completely but in distinct degree, as revealed

in the hematocrit value, which was 37.7% for the iron restricted and 45.1% for the iron supplied, and in hemoglobin content, which was 11.3g/dl in iron supplied and 9.37g/dl in iron restricted ones.

But the effect of iron administration on free protoporphyrin of erythrocyte was more clear-cut than that on hemoglobin, that is, the marked elevation of protoporphyrin in the blood cells of tumor-bearing and iron restricted rats was prevented almost completely. Free protoporphyrin in the liver showed a tendency to increase slightly by iron restriction only, and it increased more in the presence of tumor. The iron administration could protect against this elevation also completely. The appearance of the substance having absorption maximum at 493mμ in the protoporphyrin or green porphyrin fraction of the liver from tumor-bearing rats was also confirmed in this experiments. The 493mμ substance was completely suppressed by the iron supplement.

The decrease of liver catalase activity in the tumor bearer was less susceptible to the iron supplement than the lower level of hemoglobin.

Discussion

From the results described above, it is evident that the iron supplement could counteract the elevation of erythrocyte porphyrin and liver free porphyrin of tumor-bearing animals. These results supported the assumption that porphyrin accumulated in blood and liver by the impairment of iron utilization in forming heme.

The fact that the appearance of the substance with absorption maximum at 493mμ in the protoporphyrin fraction of tumor-bearing rats could be prevented completely by the iron administration indicates the possibility that this substance is produced in the liver by the impairment of iron utilization in the presence of tumor.

The protections by iron supplement against anemia and liver catalase depression were not complete. This agrees with von Euler's results (13). In this connection, Kampschmidt et al. reported that iron injection into Walker carcinoma-bearing rat corrected the anemia to the same extent as we demonstrated in this experiment, but the simultaneous apply of cobalt and iron did prevent anemia completely (14). And they also demonstrated that the supplement of iron alone or with cobalt could not overcome the decrease of liver catalase, or of cytochrome c in muscle. It is conceivable that for the formation of hemoglobin, catalase and cytochromes, besides the enough amount of iron supply, there are demanded other co-factors, which are also suppressed in tumor bearing animals. That this co-factor for hemoglobin is cobalt is evident from the results of Kampfschmidt. As the stimulating factor for cytochromes formation copper has been postulated by several workers (15). Then the simultaneous administration of copper or some other elements with iron may be expected to overcome the decrease of catalase and cytochromes of tumor-bearing animals.

Summary

The rats transplanted with Rhodamine sarcoma were fed on high iron supplied diet with the aim of correcting the symptoms presumably arising from the iron deprived state in the hosts.

Iron supplement in 2 mg dose a day adequately prevented the elevation of erythrocyte porphyrin and liver free porphyrin of tumor-bearing rats, but anemia and liver catalase depression of the tumor bearer were corrected incompletely by iron administration.

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EFFECT OF 4-NITROQUINOLINE N-OXIDE DERIVATIVES ON THE DPN DEPENDENT ENZYME SYSTEMS

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It has been reported from this laboratory that certain nitroquinoline derivatives show a distinct anti-cancer action (1) as well as powerful carcinogenic potency (2), and that there is a close parallelism between the anti-cancer action and glycolysis-inhibiting effect (3). Since these compounds have strong reactivity toward sulfhydryl groups (4), the mechanism by which they inhibit glycolysis of carcinoma cells was assumed to be due to the blocking of the glycolytic system at 3-phosphoglyceraldehyde dehydrogenase step, as in the case of monoiodoacetic acid (5). Recent studies comfirmed this early assumption by the findings that among the glycolytic intermediates hexosemono- and di-phosphate were accumulated and phosphoenolpyruvate was reduced by the addition of one of these quinoline compounds to the system (6).

In the course of these experiments we noted the works of Holtzer *et al.* (7, 8, 9) and Roitt (10), who have been studying the carcinostatic action of ethyleneimine compounds and found that these compounds also possess strong inhibiting action on glycolysis of Ehrlich ascites cells. As to the mode of glycolysis inhibiting action of the ethyleneimine compounds, they first presumed the inhibition of triosephosphate dehydrogenase, considering the sulfhydryl-attacking properties of those compounds. Experimental results showed, however, that the inhibition of triosephosphate dehydrogenase by ethyleneimine compounds was not so strong as by monoiodoacetic acid, that there was observed a drastic decrease of DPN concomitantly with the glycolysis inhibition, and that the glycolysis inhibiting action of these compounds was completely counteracted by a small excess of nicotinamide. These findings led them to assume the mechanism of glycolysis inhibition to be due to the DPN splitting or synthesis inhibiting action of these compounds.

Turning again to the mode of action of the nitroquinoline derivatives, although they have varying activities of blocking sulfhydryl groups, closer observation shows that there exist some contradictions between the affinities toward sulfhydryl group and glycolysis inhibiting actions. For example 4-nitroquinoline N-oxide has stronger affinity for sulfhydryl groups than 4-nitroquinaldine N-oxide but the latter has more marked action on glycolysis than the former, as revealed by Fukuoka *et al.* (3) and Endo (11).

These circumstances rendered it advisable for us to reexamine the mode of action

of nitroquinoline derivatives, especially in connection with the findings of Holtzer *et al.* The experiments reported here were carried out to examine the effects of nicotinamide on the glycolysis inhibiting action of nitroquinoline derivatives and on the behavior of diphosphopyridine nucleotide (DPN), and the effects of the nitroquinoline derivatives on the DPN splitting and synthesis enzyme activities of tumor cells in comparison with those of normal tissues. The effect of these compounds on the so-called sulfhydryl enzymes, such as hexokinase, succinate oxidase and α-ketoglutarate oxidase were also tested.

Methods and Materials

Tumor cells: Tumor cells used in there experiments were those of Ehrlich ascites carcinoma of the mouse, ascites Hepatoma AH 56 and Yoshida ascites sarcoma of rat. Cells were aspirated and washed 3 times with ice-cold saline and the contaminating red blood cells were separated by slow centrifugation.

Anaerobic glycolysis: Measurement of glycolysis was performed by the classical Warburg manometric method (12). In the Warburg flask was placed a total volume of 3ml of Krebs-Ringer-bicarbonate buffer solution, containing glucose 200mg/dl in the final concentration and 0.2ml of 10% tumor cells suspension in each side arm. The gas phase was 95 % N_2 and 5 % CO_2. After 10 minutes temperature equilibrium at 38°, tumor cells in the side arm were tipped into the main chamber to make contact with the other components, which included glucose and inhibitor. The rate of glycolysis was measured manometrically by the amount of evolved CO_2 generated by the lactic acid formation in bicarbonate buffer at 5 or 10 minutes intervals for two hours. For the purpose of determininig the DPN content in the glycolysing cells in the presence or absence of inhibitor, more cells were demanded than used in these routine tests, so the assay system of glycolysis was sat up on a larger scale using Thunberg tubes. In each tube 1.5ml of glucose, 400mg/dl in Krebs-Ringer-phosphate buffer of pH 7.3, was placed in the main chamber and 1.5ml of 40% Ehrlich cell suspension in the side arm with the same buffer solution. In the experiments, nitroquinoline and nicotinamide, either singlly or together, were contained in the glucose solution of each tube. The gas phase was replaced with N_2. After 10 minutes of preincubation at 38°, the cell suspention was mixed with the other solution to start glycolysis, the reaction was stopped at 20, 40 and 60 minutes by the addition of 1.0ml of 20% TCA and the DPN content of each tube was measured by the alcohol dehydrogenase system (13).

Measurement of DPN synthesizing enzyme activity: The activity of DPN synthesizing enzyme was measured by the procedure of Kornberg (14), which was modified by the authors to include fluoride as reported previously (13). The DPN content in the reaction mixture was measured by increase or decrease of absorption at 340mμ after the addition of alcohol dehydrogenase and ethanol or acetaldehyde (13).

DPNase activity: The assay of DPNase activity was carried out according to the method of Zatman *et al.* (15). The reaction mixture consisted of 0.3 ml of 0.1 M phosphate buffer pH 7.2, 1mg of oxidized DPN and enzyme (10% tissue homogenate in water) in total volume of 0.6ml. After incubation at 38° for 20 minutes, 0.6ml of 20% TCA was added and the protein removed by the centrifuge and the DPN remaining in the supernatant was measured as in the case of DPN synthesis.

Determination of succinate and α-ketoglutarate oxidase activities: The assay of succinate and α-ketoglutarate oxidase activities of rat liver mitochondria was carried out by the manometrical procedures of Green (16) with a slight modification. The reaction mixture (pH 7.4, final volume 3.0 ml) contained the following components: 0.0067M pH 7.4 K-phosphate buffer, 0.005M $MgCl_2$, 0.001 M ATP, 0.001M cytochrome C, 0.003M substrate and rat mitochondria. The mixture was incubated at 38° and the gas phase was air. The oxygen consumption was measured at 5 or 10 minutes intervals.

Isolation of mitochondria from rat liver: For the assay of succinate and α-ketoglutarate oxidase, mitochondria was isolated by the isotonic sucrose procedure of Hogeboom *et al.* (17), and 5mM EDTA was included in the sucrose solution. The 10% homogenate of rat liver was centrifuged at 600 g for 10 minutes to sediment nuclei, unbroken cells and red cells, and the supernatant was centrifuged at 8500 g for 10 minutes to sediment the mitochondria. The precipitate was washed once with 0.25M sucrose solution containing 5 mM EDTA and then suspended in the same sucrose-EDTA solution.

Assay of hexokinase activity: The assay of hexokinase reaction was carried out by the method of Boyland *et al.* (18) with slight modification. To each stoppered tube was added 0.5ml of pH 7.0 0.2M tris-HCl buffer 0.1ml of 0.2M $MgCl_2$, 1.0ml of 0.005 M glucose solution containing enzyme of about 3 Kunitz-McDonald units and 0.8ml of water or of inhibitor solution. The tubes were then incubated at 30° for 10 minutes and the reaction started by the addition of 0.5 ml 0.02 M ATP. The reaction was stopped and reaction mixtures deproteinised after 20 minutes by the addition of 3ml of 0.3N Ba $(OH)_2$ and $ZnSO_4$, and hexokinase activity was determined by the glucose remaining in the supernatants, according to the method of Nelson and Somogyi (19).

Materials: Nitroquinoline N-oxide derivatives were kindly supplied by Dr. Sakai, the Kaken Chemicals, Ltd., Tokyo. ATP (disodium salt) and DPN (90% purity) were obtained from the Sigma Co., Ltd., and yeast hexokinase (28,000 K-M unit/g at 30°) was purchased from the Nutritional Biochemicals Co., Ltd.

Results

Effect of nicotinamide on the glycolysis-inhibiting action of nitroquinoline derivatives: To analyse the mechanism of glycolysis inhibition by nitroquinoline derivatives, we used 4-nitroquinoline N-oxide in the previous report (6) but in the present

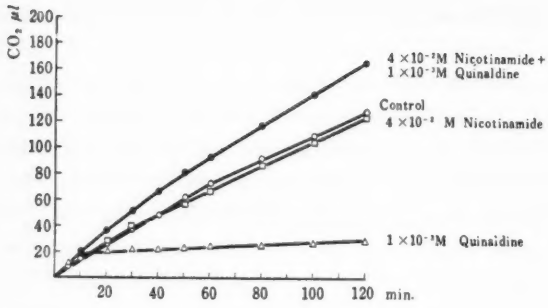

Fig. 1. Effect of Nicotinamide on the Glycolysis of Ehrlich Carcinoma Cells Inhibited by 4-Nitroquinaldine N-oxide.

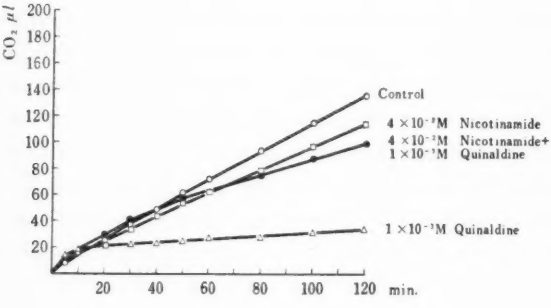

Fig. 2. Effect of Nicotinamide on the Glycolysis of Hepatoma Cells Inhibited by 4-Nitroquinaldine N-oxide.

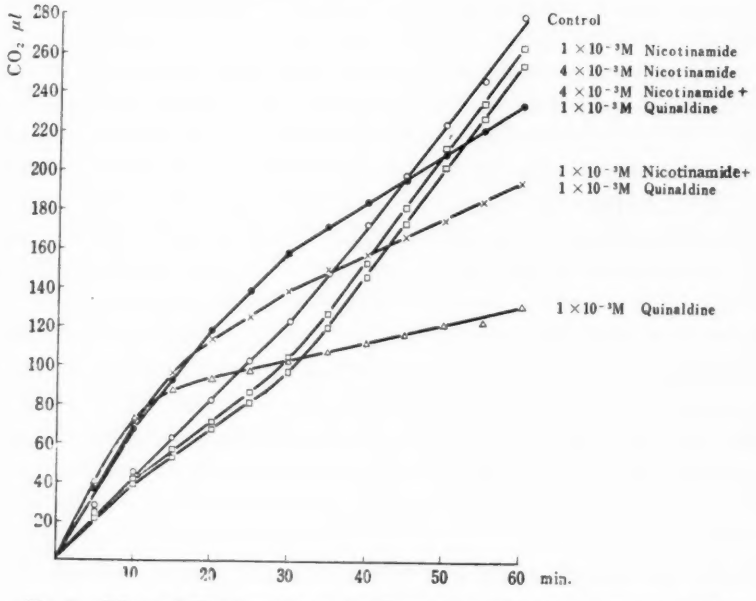

Fig. 3. Effect of Nicotinamide on the Glycolysis of Yoshida Sarcoma Cells Inhibited by 4-Nitroquinaldine N-oxide.

experiments, we chosed more potent inhibitors from the list of nitroquinoline derivatives of Fukuoka *et al.* (3) to test the effect on them of nicotinamide. As were illustrated in Figs. 1, 2 and 3, 4-nitroquinaldine-N-oxide exhibited severe inhibiting action on the anaerobic glycolysis of Ehrlich cell (Fig. 1), hepatoma AH 56 (Fig. 2) and Yoshida sarcoma (Fig. 3) at 10^{-3}M final concentration, after 10 minutes time lag. But in the presence of nicotinamide at 4×10^{-2}M final concentration the inhibiting action of the nitroquinaldine was almost completely abolished and this counteracting effect was also demonstrated by one-tenth concentration of nicotinamide. In the case of Ehrlich ascites cells, nicotinamide in higher concentration not only prevented the inhibiting action, but also some times slightly accelarated the glycolysis over the level of the control without the inhibitors, as illustrated in Fig. 1. On the hepatoma cells, nicotinamide exerted a little less effect than on Ehrilich cells (Fig. 2). And in the experiments with Yoshida sarcoma cells, the counteracting action of nicotinamide, which was used in so diluted a concentration as to be comparable to that of inhibitor, became less complete during the course of incubation, and retained the glycolysis rates at 70% of control in 4×10^{-3}M concentration and 50% in 1×10^{-3} M. Without nicotinamide only 20 percent activity was preserved as presented in Fig. 3 and Table 1.

Table 1. Effect of Nicotinamide on the Glycolysis-inhibition of 4-Nitroquinaldine-N-oxide.

Nicotinamide concentration		—	—	4×10^{-3}M	1×10^{-3}M	4×10^{-3}M	1×10^{-3}M
Nitroquinaldine concentration		—	1×10^{-3}M	1×10^{-3}M	1×10^{-3}M	—	—
15—60 min. after beginning of reaction	$CO\mu l$	20.71	4.18	14.12	9.85	20.25	20.55
	Inhibition of glycolysis %	0	79.8	31.8	52.5	2.0	0.9

4-Nitroquinaldine-N-oxide has the strongest glycolysis inhibiting action, but its reactivity to sulfhydryl groups is the lowest among the nitroquinoline N-oxide derivatives as tested by Endo (11). An additional experiment was carried out with 6-chloro-4-nitroquinoline N-oxide, which reacts to sulfhydryl group most strongly, and it was revealed that this compound produced rather less effect than 4-nitroquinaldine N-oxide on glycolysis of Ehrlich cells as reported in the previous paper (3). In this case also, nicotinamide protected completely the glycolysis against the inhibiting action of this chloro compound, as illustrated in Fig. 4.

Effect of nicotinamide on the depression of DPN in Ehrlich cells by 4-nitroquinaldine N-oxide: The effects of 4-nitroquinaldine N-oxide, nicotinamide and the mixture of both compounds on the DPN level of Ehrlich cells in anaerobic glycolytic condition are shown in Table 2. By the addition of 1×10^{-3}M 4-nitroquinaldine N-oxide in the glycolytic system, the DPN level of Ehrlich cells was decreased

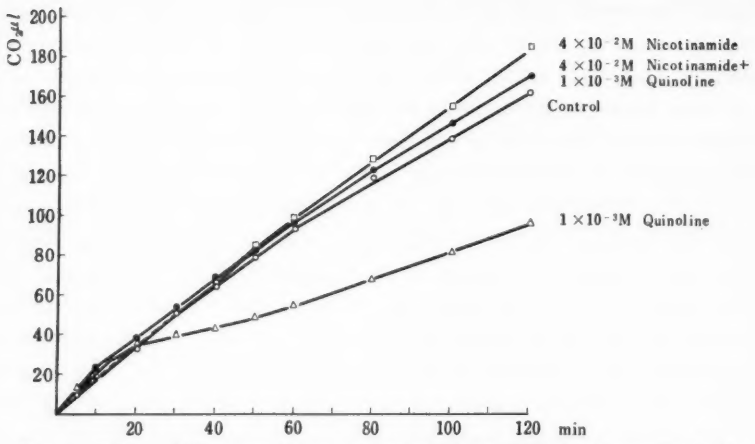

Fig. 4. Effect of Nicotinamide on the Glycolysis of Ehrlich Carcinoma Cells Inhibited by 6-Chloro-4-nitroquinoline N-oxide.

Table 2. Effect of Nicotinamide on the Decrease of DPN in Ehrlich Carcinoma Cells by 4-Nitroquinaldine N-oxide in Anaerobic Glycolytic Condition.

	0			20 min.			40 min.			60 min.		
	DPN	DPNH	Nm-S	DPN	DPNH	Nm-S	DPN	DPNH	Nm-S	DPN	DPNH	Nm-S
Control	72.3	5.0	110	89.0	0	124	68.7	0	116	74.4	0	118
+Quinaldine*				37.5	28.3	6	25.0	0	0	20.5	0	0
+Quinaldine* +Nicotinamide**				68.7	19.1	99	46.1	17.7	85	41.8	17.1	79
+Nicotinamide**				93.5	2.1	93	80.1	1.4	119	72.3	0	117

DPN γ/g tissue

Nm-S: Nicotinamide N-substituted compound measured by the flurometry of Jevitas et al. (20).

* Quinaldine at final concentration of 1×10^{-3}M

** Nicotinamide at final concentrationof 1×10^{-2}M

suddenly, and at the same time DPNH became detectable, and pyridine nucleotide, measured as the nicotinamide N-substituted compound (Nm-S) by fluorometry (20), was depressed more drastically than measured by alcohol dehydrogenase as DPN and DPNH, and in the later period of incubation it vanished absolutely. This depression was prevented considerably by the addition of 4×10^{-2}M nicotinamide, but the tendency for DPNH to increase was not counteracted by the addition of nicotinamide.

Effect of 4-nitroquinaldine N-oxide on the DPN synthesizing and splitting enzyme systems of various tissues. The addition of 4-nitroquinaldine N-oxide to the DPN synthesizing system of normal tissue homogenate, scarely affected the enzyme activities,

Table 3. Effect of 4-Nitroquinaldin N-oxide on the DPN Synthesizing Activities of Various Tissues (DPN μM/hr./g tissue)

(Tissue)	Mouse		Rat	
	Control	+Quinaldine	Control	+Quinaldine
Liver	7.86	9.65	3.26	2.44
Spleen	6.12	5.07	1.05	1.05
Kidney	4.98	6.21	1.77	1.34
Ehrlich	2.58	1.72		
Yoshida			1.34	1.05
Hepatoma			1.53	0.38

as shown in Table 3. By the addition of the compound, the DPN synthesizing activities of Ehrlich and Yoshida cells were decreased by 33 and 21% respectively. In the case of hepatoma the supression amounted to 75% of that of the control.

The effect of nitroquinaldine N-oxide on the DPNase activities of normal tissues was also very slight, except in the case of rat kidney, in which the activity was inhibited to the half level of the control, as shown in Table 4. The DPNase activities of Ehrlich and Yoshida cells were decreased to about 50 and 30%, respectively, of the controls, but the hepatoma DPNase was activated slightly over that of control, by the addition of 4-nitroquinaldine N-oxide.

Table 4. Effect of 4-Nitroquinaldine N-oxide on DPNase of Various Tissues (Split DPN μM/20 min/g tissue)

(Tissue)	Mouse		Rat	
	Control	+Quinaldine	Control	+Quinaldine
Liver	16.9	17.5	13.4	11.3
Spleen	17.9	17.5	25.0	25.9
Kidney	18.0	15,3	6.2	2.6
Ehrlich	4.8	2.6		
Yoshida			13.5	10.0
Hepatoma			5.9	7.0

Effect of 4-nitroquinaldine N-oxide on the activities of hexokinase, succinate oxidase and α-ketoglutarate oxidase. As quoted repeatedly in previous sections, nitroquinoline derivatives have considerable reactivities with sulfhydryl compounds, but there has been lacking the experimental evidence to show their inhibiting actions on the so-called sulfhydryl enzymes, which have the essential sulfhydryl groups in the molecule for their enzyme actions. Among the sulfhydryl enzymes, we selected the typical ones, such as hexokinase from yeast and succinoxidase of rat liver mitochondria, which are not DPN dependent, and α-ketoglutarate oxidase of the mitochondria

as an example of the DPN-dependent sulfhydryl enzyme. The effect of 4-nitroquinaldine N-oxide on their activities was tested.

Contrally to our early expectation, the hexokinase activity of yeast was not inhibited by 4-nitroquinaldine N-oxide even in the concentration as high as 1×10^{-3}M which is enough to cause complete glycolysis inhibition. This is shown in Table 5.

Table 5. Effects of 4-Nitroquinaldine N-oxide and p-Chrolomercuri-benzoate on Hexokinase Activity (PCMB)

Final concentration	Phosphorylated glucose amount γ/20min	Relative activity
0	561	100
Quinaldine 1×10^{-3}M	626	117
Quinaldine 1×10^{-4}M	599	106.8
Quinaldine 1×10^{-5}M	599	106.8
0	591	100
PCMB 1×10^{-3}M	0	0.0
PCMB 1×10^{-4}M	17	3.0
PCMB 1×10^{-5}M	165	28.0

But under the same condition hexokinase could be inhibited perfectly by the addition of 1×10^{-3}M p-chloromercuribenzoate, which is fairly assumed to be the most typical sulfhydryl blocking reagent.

As illustrated in Fig. 5, 4-nitroquinaldine N-oxide exhibited also no effect on the succinate oxidase of the rat liver mitochondria.

In contrast to these negative results, the α-ketoglutarate oxidase activity of the rat liver mitochondria was inhibited severely by the addition of 4-nitroquinaldine N-oxide. But this inhibition was not hindered by the addition of nicotinamide or DPN as shown in Figs. 6 and 7. For unknown reason, in this experiment, the nicotinamide in 4×10^{-2}M concentration depressed the α-ketoglutarate oxidase activity to about half of that of the control (Fig. 6).

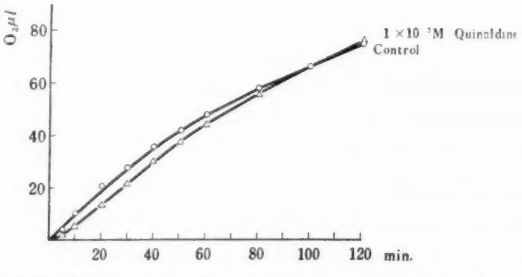

Fig. 5. Effect of 4-Nitroquinaldine N-oxide on Succinate Oxidase of Rat liver Mitochondria.

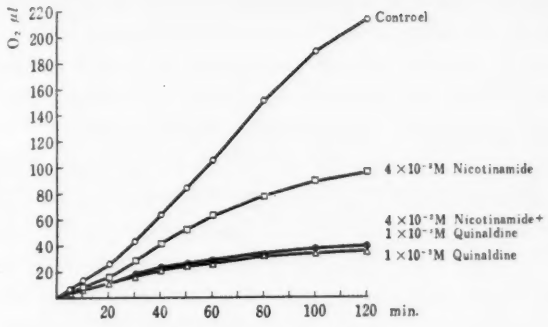

Fig. 6. Effect of Nicotinamide on α-Ketoglutarate Oxidase of Rat Liver Mitochondria Inhibited by 4-Nitroquinaldine N-oxide.

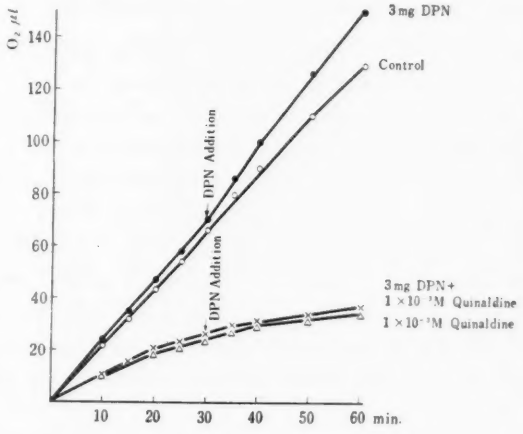

Fig. 7. Effect of DPN on α-Ketoglutarate Oxidase of Rat Liver Mitochondria Inhibited by 4-Nitroquinaldine N-oxide.

Discussion

In the experiments described above, it is clearly demonstrated that the glycolysis-inhibition by nitroquinoline derivatives is almost completely counteracted by slight excess of nicotinamide, and the very drastic depression of the DPN level in the glycolysis-inhibited tumor cells is also prevented nearly completely by the addition of nicotinamide. These results with nitroquinoline derivatives are quite similar to those of glycolysis inhibition by ethyleneimine derivatives reported by Holtzer *et al.* and Roitt and suggest that the mode of glycolysis inhibition by these two classes of molecules might be alike.

The findings that 4-nitroquinaldine-N-oxide inhibited only the DPN-dependent α-ketoglutarate oxidase, among the so-called sulfhydryl enzymes tested in these experiments, such as succinoxidase and hexokinase, also afforded the evidence that this compound exerts its action by disturbing the mechanism of maintaining DPN level in the system, but not by its sulfhydryl attacking property.

It must be pointed out, however, that even if glycolysis is inhibited by nitroquinoline derivatives through their DPN depressing action, 3-phosphoglyceraldehyde dehydrogenase, which is DPN-dependent, must be blocked and therfore the same pattern of glycolytic intermediates as reported in the previous paper (6) will result, that is, hexose mono- and diphosphate accumulate and phosphoenolpyruvate decrease.

About the influence of 4-nitroquinoline-N-oxide on the DPN dependent enzyme systems Jackson and Lightbown (21) already reported that 4-hydroxy-2-alkylquinoline N-oxide inhibited the DPNH oxidizing enzyme systems specifically. So the finding that DPNH became detectable in our experiments on the effect of 4-nitroquinaldine-N-oxide on depressing total DPN level in the cell, might be explained as being due to the same action of 4-nitroquinaldine N-oxide as that of quinoline-N-oxide compounds tested by Jackson and Lightbown.

In the experiment to clarify the mode of depressing DPN level in the glycolysis system, some distinct inhibitory effects of nitroquinoline derivatives on the DPN synthesising enzyme activity were detected especially in tumor cell preparation, but these inhibitory actions of nitroquinoline were too mild to be considered as the cause of the severe depressed of DPN in the cells. The effect of nitroquinoline on DPN splitting system was rather slight, though of inhibiting nature, and the fact nitroquinoline activates the DPN splitting enzyme was not confirmed at all. However, the DPN splitting enzyme might be supposed to be inactive in untreated intact cells as in the case of alkaline-phosphatase and ATPase, so there remains the possibility that the experiments carried out with water-disrupted cell homogenates were unsuitable to detect the effect of nitroquinoline on the DPN splitting enzyme activity in the intact cells.

In the mitochondrial preparation the inhibition of α-ketoglutarate oxidase by 4-nitroquinaldine N-oxide could not be prevented by nicotinamide, nor be counteracted by the addition of DPN itself, after the inhibition has been established completely. Several reasons are conceivable to account for the discrepancy between intact cells and mitochondrial preparation in the protecting effect of nicotinamide. Nicotinamide may be supposed to be effective in protecting glycolysis by inhibiting DPNase, which is accelarated by nitroquinoline. The failure of nicotinamide to protect α-ketoglutarate oxidase might suggest that in mitochondria system α-ketoglutarate oxidase is inhibited not by the DPN depression caused by nitroquinoline but by the retardation of DPNH oxidation as revealed by Jackson and Lightbown. The failure to restore α-ketoglutarate oxidase activity by DPN itself might be due also to the permeability of mitochondria membranes and the specificity of binding sites of DPN. In this connection, Hunter *et al.* (22) demonstrated recently that reincorporation of DPN into phosphate-swollen rat liver mitochondria can occur in the presence of ATP, but exposure of the mitochondria to ATP before exposure to DPN prevents reincorporation.

About the mechanism of depressing DPN level by 2 : 4 : 6-triethylene-imine-1 : 3 : 5-triazine (TEM) *in vitro* Roitt expressed the view that the compound elevates the DPN splitting enzyme. Holtzer *et al.* also prefered the view that quinone-ethyleneimine compounds evelevate the DPN splitting enzyme to that of inhibiting DPN synthesis. But Waravdekar *et al.* (23) insisted that, as far as they tested, carcinostatic drugs depressed DPN levels of tumore tissues *in vivo* by inhibiting DPN synthesising enzyme, not by the activation of DPN splitting enzyme. The conclusive evidences have not been obtained to decide whether the nitroquinoline derivatives, in the depression of DPN, act by the mechanism proposed by Roitt as well as Holtzer for the ethyleneimine derivatives, or that proposed by Waravdekar. Since in the control experiment without nitroquinoline nicotinamide did not elevate the DPN level in the cell, the protecting action of nicotinamide may fairly be assumed to be due to inhibiting the increased of DPNase activity caused by nitroquinoline. And the finding in our experiments that nitroquinoline inhibited more strongly the DPN synthesising enzyme of the tested tumor cells than of normal tissues supported the particularity of DPN metabolic system in tumor cell as suggested by Waravdekar, who showed that tumor damaging drugs depressed the DPN synthesis enzyme of only tumor cells, but not of normal tissues *in vivo*.

We could confirm that nitroquinoline did not react with DPN non-enzymatically at all, but in this connection, Kaplan *et al.* (24) reported that nicotinamide analogue, such as isonicotinic acid hydrazid could replace the nicotinamide moiety of DPN by the action of enzyme, namely, isonicotinic acid hydrazid-insensitive DPNase, and then abolish the DPN activity. Examination of the possibility that nitroquinoline derivatives used in this experiment might react with DPN enzymatically is now being attempted in this laboratory.

Summary

The anerobic glycolysis of the cancer cells was inhibited by nitroquinoline N-oxide derivatives, but the extents of glycolysis-inhibition were not closely proportional to the reaction of these substances with the sulfhydryl group. The inhibition of glycolysis by nitroquinoline derivatives was prevented by the addition of nicotinamide, and these compounds decreased the DPN level of the inhibited cell, as was reported in the case of ethyleneimine compounds by Roitt as well as Holzer *et al.*

The depression of DPN level by nitroquinoline derivatives was also counteracted completely by nicotinamide. The dffects of nitroquinoline derivatives on the DPN synthesizing and splitting enzyme systems of various tissues were tested and it was revealed that the both enzyme activities especially of cancer cells were inhibited. But the grades of these inhibitions were not so dramatic as was of the glycolysis, and the significance of these inhibitions remains as an open question.

Among the sulfhydryl enzymes tested in these experiments, the nitroquinoline N-oxide inhibited the DPN-dependent α-ketoglutarate oxidase but had no interference with DPN-nondependent succinate oxidase and hexokinase activities. But the inhibition of α-ketoglutarate oxidase by nitroquinoline N-oxide was not prevented by the addition of nicotinamide or DPN itself.

From these results it may be concluded that nitroquinoline derivatives inhibit glycolysis by depressing DPN level as in the case of ethyleneimine compounds but not of monoiodoacetate, which latter inhibits glycolysis by blocking sulfhydryl enzyme such as glyceraldehyde dehydrogenase.

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